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13. ABSTRACT ***(Maximum 200 Words)***

Vascular endothelial growth inhibitor (VEGI), an novel cytokine of the TNF-superfamily, is produced predominantly by endothelial cells and exhibits potent anti-angiogenic and anti-cancer activities (Zhai et al., Int. J. Cancer, 82:131, 1999). We report here that the effect of VEGI on endothelial cells is cell-cycle dependent: it mediates an early G1 arrest in quiescent cells, but induces apoptotic death in proliferating cells. VEGI inhibits DNA synthesis in G0-synchronized adult bovine aortic endothelial (ABAE) cells, which do not express VEGI themselves. The inhibition was reversible once VEGI was removed from the culture media. VEGI treated G0-cells lacked typical markers of late G1 phase, such as the hyperphosphorylation of the retinoblastoma gene product (pRB) and the upregulation of the c-myc gene, suggesting an early G1 arrest. In contrast, exposure of ABAE cells of logarithmic growth phase to VEGI resulted in apoptotic cell death. Consistently, VEGI expression in human umbilical cord vein endothelial cells (HUVEC) was found to be markedly upregulated in confluent cells when compared to proliferating cells. These findings support the view that VEGI may play a role in the maintenance of the low turn over rate of the endothelium of an established vasculature.

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INTRODUCTION

The endothelium is the major component of the vascular system. It plays an important role in many vascular functions, including tissue homeostasis, fibrinolysis and coagulation, blood-tissue exchange, and neovascularization (1). The endothelium in a mature vasculature under physiological conditions is mostly a quiescent tissue (2). Maintenance of the quiescence of the endothelium is likely to involve the suppression of endothelial cell proliferation and the elimination of excessive endothelial cells. Endothelial cells become highly proliferative under certain conditions. The proliferation of endothelial cells in physiological angiogenesis as seen *in utero*, in wound healing, or in the female reproductive system, is apparently well-controlled by a balance between positive and negative regulators of angiogenesis (3, 4). In contrast, pathological angiogenesis as seen in cancer, rheumatoid arthritis, and several other important diseases, is characterized by prolonged neovascularization with no termination. A negative regulation mechanism that functions in a physiological setting thus appears to be missing or poorly functioning in a pathological setting. A number of naturally existing inhibitors of angiogenesis have been reported. These include thrombospondin (5), platelet factor-4 (6), angiostatin (7), and endostatin (8). In addition, many growth factors and cytokines, such as platelet-derived growth factor (9), transforming growth factor-β (10), angiopoietin-1 (11), and angiopoietin-2 (12), have been shown to be involved in the stabilization and maturation of new blood vessels through modulations of endothelium-periendothelial cell interactions. However, the molecular mechanism underlying the maintenance of the extremely low turn over rate of the endothelial cells in a normal vasculature remains largely unclear.

We have recently reported the discovery of an endothelial cell-specific gene product, vascular endothelial cell growth inhibitor (VEGI) (13, 14). The protein consists of 174-amino acids, with a 20-30% sequence homology to members of the TNF superfamily. Northern blotting analysis of a wide variety of cell lines and primary cell cultures indicates that the VEGI gene is expressed predominantly in endothelial cells. Additionally, the VEGI mRNA is detectable in many adult human organs, suggesting a physiological role of the gene in a normal vasculature. The function of VEGI was examined in a number of cellular and animal models. Recombinant VEGI inhibited endothelial cell proliferation with a remarkable potency, but had no effect on the growth of any other types of cells examined. The protein also inhibited the formation of capillary-like structures by endothelial cells in collagen gels, and the growth of capillaries into collagen gels placed on the chick chorioallantoic membrane. Overexpression of a secreted form of VEGI in murine colon cancer cells (MC-38) strongly inhibited the ability of these cells to grow tumors in syngenic C57/BL mice. Moreover, co-inoculation of human breast cancer cells with Chinese hamster ovary cells overexpressing VEGI led to marked inhibition of the growth of the breast cancer xenograft tumors in nude mice.

We report here that the effect of VEGI on endothelial cells is cell-cycle dependent. Treatment of G_0-synchronized adult bovine aortic endothelial (ABAE) cells with VEGI gave rise to a growth arrest at the early G_1-phase of the cell cycle. On the other hand, exposure of proliferating ABAE cells to VEGI resulted in apoptotic cell death. In addition, VEGI expression in proliferating human umbilical cord vein endothelial cells (HUVEC) was markedly upregulated in confluent cells as compared to that in growing cells. Based on these findings, we propose that this cytokine may play a physiologically significant regulatory role as a suppressor of neovascularization in the maintenance of the quiescence of an established vasculature.

BODY

VEGI-Treatment of G_0-Synchronized Endothelial Cells Causes a Reversible Growth Arrest: We investigated whether VEGI can suppress the initiation of endothelial cell proliferation in response to growth stimuli. ABAE cells were used in this study because these cells do not express VEGI but are highly responsive to this cytokine. The cells were synchronized in the G_0-phase of the cell cycle by cell-cell contact inhibition in confluent cultures. Once re-seeded in culture media, the cells re-entered the growth cycle. The ability of these cells to synthesize DNA was determined in the presence of various VEGI concentrations. Cells treated with VEGI were unable to incorporate ^{3}H-thymidine (Fig. 1A). The inhibition was dose-dependent, with a half-maximum inhibition concentration (IC_{50}) of about 30 ng/ml (1.5 nM). VEGI thus prevented the G_0-cells to advance into the S-phase. Determination of the number of cells indicated that the G_0-cells began to undergo the first mitotic division in about 20 hours following seeding. The cell number doubled once after about 40 hours (Fig. 1B). The growth rate was lowered to about 18% over the same period of time when the cells were cultured in the presence of VEGI at 60 ng/ml. This is close to the 25% increase expected when an inhibitor is present at a concentration 2-times of the IC_{50} value. Moreover, the cells resumed growth at a rate comparable to that of the untreated cells once VEGI was removed from the culture media (Fig. 1C). The growth inhibition by VEGI was thus reversible.

VEGI Induces Early G_1 Growth Arrest: We then determined the effect of VEGI treatment on the occurrence of typical markers of the G_1-phase of the cell cycle. A well-established marker for the late hours of the G_1 phase is the hyperphosphorylation of the retinoblastoma gene product, pRB, which is underphosphorylated in G_0 or early G_1 cells (15). To determine the phosphorylation status of the pRB protein, G_0-synchronized ABAE cells were seeded in the presence or absence of VEGI (60 ng/ml). The cells were harvested at various time intervals over a 3-day period, and subjected to Western blotting analysis, using a monoclonal antibody against pRB. The occurrence of a predominant, higher molecular weight species of pRB (Fig. 2) indicated that the protein was mostly hyperphosphorylated within 24 hours following seeding in the absence of VEGI, whereas in VEGI treated cells the pRB protein was still mostly underphosphorylated, as represented by the lower molecular weight species (Fig. 2B), and remained underphosphorylated as long as VEGI was present in the culture media. Thus, the growth arrest caused by VEGI treatment precedes pRB phosphorylation which occurs in the late G_1 phase. To further confirm this finding, we determined the expression of the *c-myc* gene, another well-studied late G_1 marker (16). It was clear that, determined by Western blotting analysis of the Myc protein, *c-myc* gene expression in ABAE cells following VEGI treatment was inhibited (Fig. 2). The Myc protein level was elevated in the proliferating cells as expected, but was nearly depleted in G_0-synchronized ABAE cells cultured in the presence of VEGI for 72 hours. These data indicate that treatment of G_0-synchronized ABAE cells with VEGI gives rise to an early G_1 growth arrest.

VEGI Treatment Induces Cell Death of Proliferating ABAE Cells, But Not Quiescent Cells: We noticed during the course of the study that a variable number of cells were lost from the cultures following VEGI treatment, and realized that endothelial cells of different growing status responded to VEGI differently. We therefore compared the effect of VEGI on ABAE cells that have entered the growth cycle with that on quiescent cells. As mentioned earlier, G_0-synchronized ABAE cells upon re-seeding enter into the cell cycle and start the first mitotic

division within about 20 hours. The cell number doubles approximately every 20 hours until the cells are confluent once again in approximately 6 days, at which time most of the cells are quiescent (Fig. 3A). VEGI was added to the cell cultures at various intervals during this period of time, namely, at the beginning when the G_0-cells were being re-seeded, in the middle of the logarithmic growth phase of the cells in culture, and at the end when the cells were nearly confluent (arrows, Fig. 3A). The cell numbers were determined in 24-hour intervals following each addition of VEGI. As expected, the cell numbers remained largely unchanged when the freshly seeded G_0-synchronized ABAE cells were treated with VEGI. Cell number did not change in confluent cultures either. However, an marked loss of cells was found when the cultures were treated with VEGI on day 2 and day 4 following seeding, The cells at these time points were in the logarithmic phase of growth. These results indicate that, besides the induction of an early G_1 growth arrest of quiescent cells responding to growth stimuli, VEGI can also induce death of ABAE cells that have entered the cell cycle when they are exposed to this growth inhibitor.

Apoptosis Is the Cause of VEGI-Induced Death of Proliferating ABAE Cells: To determine whether programmed cell death was the cause of VEGI-induced death of the proliferating ABAE cells, the extent of nuclear DNA fragmentation in these cells was analyzed. G_0-synchronized ABAE cells were placed in culture. VEGI (60 ng/ml) was added to the media either at the time of seeding (day 0), on day 2, or on day 6. The cells were harvested in 48 hours following the addition of VEGI. The cells were treated with bromodeoxyuridine (BrdU) in order to label the 3'-hydroxyl ends of fragmented DNA to identify apoptotic cells. The cells were then subjected to fluorescent-activated cell sorting (FACS) (Fig. 4A). It was found that the percentage of the cells with extensive nuclear DNA fragmentation was significantly higher in cells treated with VEGI on day 2, when the cells were undergoing proliferation, as compared to that in cells treated either on day 0 at the time when the G_0-synchronized cells were being re-seeded, or on day 6 when the cell cultures were nearly completely confluent (Fig. 4A). The percentage of apoptotic cells in the cell cultures treated with VEGI on day 0, day 2, and day 6 were 5.8%, 20.8%, and 7.6%, respectively, when analyzed in 24 hours following the addition of VEGI to the culture media (Fig. 4B). These death rates are likely to account for most of the loss of cells when proliferating ABAE cells were treated with VEGI (Fig. 3). These results suggest that VEGI induces programmed death only in proliferating ABAE cells, but not in cell resting in G_0.

The VEGI-mediated cell cycle-dependent apoptosis was further investigated by using a different method. Again, G_0-synchronized ABAE cells were treated with VEGI on day 0, day 2, and day 6. Fragmented DNA molecules in apoptotic cells were identified in 48 hours following VEGI treatment by using *in-situ* end-labeling with biotinylated UTP, then visualized with avidin-conjugated horseradish peroxidase. ABAE cells treated on day 2 displayed purple-colored discreet apoptotic bodies because of DNA fragmentation (Fig. 5C). The apoptotic bodies were rare in untreated cells, as well as in cells treated on day 0 and day 6 (Figs. 5A, 5B, 5D). These data confirm that VEGI induces programmed cell death occurred only in endothelial cells that are undergoing proliferation.

KEY RESEARCH ACCOMPLISHMENTS

Discovered that VEGI has a dual role in the regulation of endothelial cell biology: 1) VEGI acts as a growth suppressor to prevent G0 endothelial cells to re-enter the growth cycle;

this role is probably a key in the maintenance of the quiescence of an established blood vessel; 2) VEGI induces programmed cell death to endothelial cells that are undergoing proliferation; this role is probably important in the termination of angiogenesis.

REPORTABLE OUTCOMES

Manuscripts:

Zhai Y, Ni, J, Jiang, GW, Lu, J, Xing, L, Lincoln, C, Janat, F, Kozak, D, Rojas, L, Aggarwal, BB, Ruben, S, Li, LY, Gentz, R, and Yu, GL. VEGI, a novel cytokine of the TNF family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas in vivo. The FASEB Journal, *13:* 181-189, 1999

Zhai, Y, Yu, JY, Iruela-Arispe, L, Huang, WQ, Wang, Z, Hayes, AJ, Lu, J, Jiang, GW, Rojas, L, Lippman, ME, Ni, J, Yu, GL, and Li, LY. A novel cytokine of the TNF superfamily is a negative regulator of angiogenesis. Int. J. Cancer, 82:131-136, 1999

Yu, JY, Huang, WQ, Hayes, A, Pan HG, Tian, S, and Li, LY. Modulation of Cell Cycle-Dependent Endothelial Cell Growth Arrest and Apoptosis by VEGI. Submitted 2000

Patent:

Pan, HG, Yu, JY, Li, LY. Isoforms of VEGI, an endothelial cell-specific suppressor of angiogenesis and tumorigenesis. 2000 (filed)

Recent Abstracts:

1. Yu, JY, Zhao QH, Hayes, AJ, Lippman, ME, and Li, LY: Anticancer Therapeutic Potential of VEGI. NCI SPORE Workshop, Rockville, MD, 1999
2. Yu, J, Huang, W, Hayes, A, Pan, HG, and Li, LY: A dual role of VEGI, an endothelial cell-specific gene product and anti-angiogenic factor, in endothelial cell cycle control: mediation of growth arrest of G0 cells but apoptosis of proliferating cells. AACR Annual Meeting, San Francisco, CA, 2000
3. Pan, HG, Huang, W, Yu, J, Li, LY: Identification of a secreted form of VEGI, an endothelial cell-specific antiangiogenic factor involved in negative regulation of endothelial cell proliferation. San Francisco, CA, 2000
4. Huang, W, Tian, S, Pan, HG, and Li, LY: Inhibition of breast cancer xenograft tumorigenesis by VEGI, an endothelial cell-specific anti-angiogenic cytokine of the TNF superfamily. San Francisco, CA, 2000

CONCLUSIONS

Our data demonstrated that VEGI induces two distinct cellular activities in ABAE cells: suppression of the re-entry of G_0 cells into the cell growth cycle, or programmed death to cells

that have already entered the cycle. Previously we reported that VEGI is predominantly expressed in endothelial cells, that the VEGI mRNA is readily detectable in many human organs, and that VEGI apparently acts only endothelial cells (13, 14). Together these data strongly support the view that VEGI is an endothelial cell-specific negative regulator of blood vessel growth. The physiological function of this unique cytokine is probably to suppress the growth of endothelial cells in an mature vasculature.

VEGI-induced growth arrest takes place in the early G_1-phase of the cell cycle. There are two distinguishable periods in the G_1 phase: early and late G_1. The entering of G_0 cells to early G_1 is reversible (17). Once the cells proceed to the late G_1 phase, they are obliged to continue into the S-phase. Since the VEGI treated G_0 cells were unable to incorporate ^{3}H-thymidine, the cells were unable to proceed to S-phase. We therefore analyzed the phosphorylation pattern of the pRB protein in ABAE cells in response to VEGI. The pRB protein is a key player in the regulation of G_1/S transition (15). The pRB protein undergoes a readily discernible chemical modification in a well-defined window of time. Through the preceding hours of in G_1, pRB is in an underphosphorylated form. During the last hours of G_1, most of the pRB molecules become hyperphosphorylated. Our data demonstrated that the pRB protein did not undergo hyperphosphorylation in the presence of VEGI, indicating that the cells had not reached the late G_1-phase. Hyperphosphorylation of pRB leads to the inactivation of the growth inhibitory effect of this protein (18), presumably because the hyperphosphorylated pRB protein can no longer form a complex with the E2F family of transcription factors (19). The pRB-E2F complex actively suppresses the transcription of cell cycle genes. Moreover, pRB appears to be an integrator of both positive and negative signals. Factors that promotes cell proliferation should encourage pRB phosphorylation, whereas growth inhibitory signals, such as TGF-β and cell-cell contact, prevent pRB phosphorylation and thus block the progression of cell growth into late G_1 (20). VEGI may act in a similar manner for endothelial cells.

Another G_1 marker is the Myc protein, a product of the early response gene c-*myc* whose transcription indicates the transition of the late G_1-phase to the S-phase (21). Myc is a positive regulator of G_1-specific cyclin-dependent kinase. The *c-myc* gene is activated by mitogenic signals, and is suppressed by growth inhibitory and differentiation signals. Additionally, the *c-myc* gene is subject to regulation by the pRB-E2F negative control mechanism (22). The binding of the pRB-E2F complex to the promoter of *c-myc* prevents transcription of the gene. Hyperphosphorylation of pRB leads to the dissociation of the pRB-E2F complex, which in turn permits the transcription of the target gene. Our data demonstrated that the *c-myc* gene expression is markedly upregulated in ABAE cells as they underwent the transition from G_0 to G_1 and subsequently entered the growth cycle. In the presence of VEGI, however, the Myc protein gradually diminished. This finding again indicates that VEGI prevents G_0-cells from advancing to late G_1-phase. In addition, the diminishing of Myc in VEGI treated G_0 cells is consistent with the low apoptosis rate of these cells, since Myc is an essential player in the regulation of cell growth, differentiation, and death (16, 23).

It is interesting that VEGI-induced apoptotic death of ABAE cells takes place only when these cells are undergoing proliferation. It has been reported previously that treatment of bovine pulmonary artery endothelial cells with a similar preparation of VEGI led to apoptosis (24). Cells at subconfluent densities were used in that study. Since the majority of cells in a subconfluent culture have not exited the cell cycle, the results are consistent with our findings. In the light that VEGI is expressed in the endothelium of many human organs, it is unlikely that this cytokine would induce apoptotic death to the quiescent cells in a normal endothelium. We found that no

apoptosis was observed in G_0-synchronized ABAE cells treated with VEGI. The cytokine also did not cause apoptosis to G_0 cells in confluent cultures where the cell growth was inhibited by cell-cell contact. Additionally, the growth suppression of G_0-cells was reversed once VEGI was removed from the culture media. Only when the cells had entered the growth cycle, exposure to VEGI then gave rise to apoptotic cell death. It remains to be seen whether similar observations can be made with other types of endothelial cells in culture.

The balance between proliferation and apoptosis of endothelial cells is likely to be the subject of a complicated regulatory mechanism. In addition to the activation of negative regulators and the down-regulation of positive regulators or their receptors, other factors such as signaling from the extracellular matrix acting through the integrins and endothelium-pericytes interactions (25-27), may also contribute to the regulation. The role of cytokines and growth factors in the suppression and termination of angiogenesis has only begun to be understood. Our findings provide new insights into a mechanism that potentially enables endothelial cells to negatively regulate their own proliferation and to promote programmed death. This mechanism could play an essential role in the maintenance of the quiescence of the endothelium of an established vasculature, in the termination of neovascularization, and in the regression of excessive blood vessels.

REFERENCES

1. Risau, W. Mechanisms of angiogenesis, Nature. *386:* 671-4, 1997.
2. Engerman, R. L., Pfaffenbach, D., and Davis, M. D. Cell turnover of capillaries, Lab Invest. *17:* 738-43, 1967.
3. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease, Nat Med. *1:* 27-31, 1995.
4. Hanahan, D. and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis, Cell. *86:* 353-64, 1996.
5. Good, D. J., Polverini, P. J., Rastinejad, F., Le Beau, M. M., Lemons, R. S., Frazier, W. A., and Bouck, N. P. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin, Proc Natl Acad Sci U S A. *87:* 6624-8, 1990.
6. Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., Carson, H. F., and Sharpe, R. J. Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides, Science. *247:* 77-9, 1990.
7. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma [see comments], Cell. *79:* 315-28, 1994.
8. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth, Cell. *88:* 277-85, 1997.
9. DiCorleto, P. E. and Bowen-Pope, D. F. Cultured endothelial cells produce a platelet-derived growth factor- like protein, Proc Natl Acad Sci U S A. *80:* 1919-23, 1983.

10. Antonelli-Orlidge, A., Saunders, K. B., Smith, S. R., and D'Amore, P. A. An activated form of transforming growth factor beta is produced by cocultures of endothelial cells and pericytes, Proc Natl Acad Sci U S A. *86:* 4544-8, 1989.
11. Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N., and Yancopoulos, G. D. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis [see comments], Cell. *87:* 1171-80, 1996.
12. Maisonpierre, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T. H., Papadopoulos, N., Daly, T. J., Davis, S., Sato, T. N., and Yancopoulos, G. D. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis [see comments], Science. *277:* 55-60, 1997.
13. Zhai, Y., Ni, J., Jiang, G., Lu, J., Xing, L., Lincoln, C., Carter, K. C., Janat, F., Kozak, D., Xu, S., Rojas, L., Aggarwal, B. B., Ruben, S., Li, L., Gentz, R., and Yu, G. VEGI, a novel cytokine of the tumor necrosis factor family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas in vivo [In Process Citation], Faseb J. *13:* 181-9, 1999.
14. Zhai, Y., Yu, J, Iruela-Arispe, L, Huang, W, Wang, Z, Hayes, A, Lu, J, Jiang, GW, Rojas, L, Lippman, ME, Ni, J, Yu, GL, Li, LY Inhibition of angiogenesis and breast cancer xenograft tumor growth by VEGI, a novel cytokine of the TNF superfamily, Int. J. Cancer. *82:* 131-136, 1999.
15. Weinberg, R. A. The retinoblastoma protein and cell cycle control, Cell. *81:* 323-30, 1995.
16. Amati, B., Alevizopoulos, K., and Vlach, J. Myc and the cell cycle, Front Biosci. *3:* D250-68, 1998.
17. Pardee, A. B. G1 events and regulation of cell proliferation, Science. *246:* 603-8, 1989.
18. Cobrinik, D., Dowdy, S. F., Hinds, P. W., Mittnacht, S., and Weinberg, R. A. The retinoblastoma protein and the regulation of cell cycling, Trends Biochem Sci. *17:* 312-5, 1992.
19. Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. The E2F transcription factor is a cellular target for the RB protein, Cell. *65:* 1053-61, 1991.
20. Wang, J. Y., Knudsen, E. S., and Welch, P. J. The retinoblastoma tumor suppressor protein, Adv Cancer Res. *64:* 25-85, 1994.
21. Marcu, K. B., Bossone, S. A., and Patel, A. J. myc function and regulation, Annu Rev Biochem. *61:* 809-60, 1992.
22. Oswald, F., Lovec, H., Moroy, T., and Lipp, M. E2F-dependent regulation of human MYC: trans-activation by cyclins D1 and A overrides tumour suppressor protein functions, Oncogene. *9:* 2029-36, 1994.
23. Henriksson, M. and Luscher, B. Proteins of the Myc network: essential regulators of cell growth and differentiation, Adv Cancer Res. *68:* 109-82, 1996.
24. Yue, T. L., Ni, J., Romanic, A. M., Gu, J. L., Keller, P., Wang, C., Kumar, S., Yu, G. L., Hart, T. K., Wang, X., Xia, Z., DeWolf, W. E., Jr., and Feuerstein, G. Z. TL1, a novel tumor necrosis factor-like cytokine, induces apoptosis in endothelial cells. Involvement of activation of stress protein kinases (stress-activated protein kinase and p38 mitogen-activated protein kinase) and caspase-3-like protease, J Biol Chem. *274:* 1479-86, 1999.
25. George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H., and Hynes, R. O. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin, Development. *119:* 1079-91, 1993.

26. Brooks, P. C., Clark, R. A., and Cheresh, D. A. Requirement of vascular integrin alpha v beta 3 for angiogenesis, Science. *264:* 569-71, 1994.
27. Bader, B. L., Rayburn, H., Crowley, D., and Hynes, R. O. Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins, Cell. *95:* 507-19, 1998.

FIGURE LEGEND

Figure 1. Reversible growth inhibition of ABAE cells by VEGI. Panel A: Inhibition of ^{3}H-thymidine incorporation. G_0-synchronized ABAE cells were re-seeded, in triplicate, in the presence of indicated concentrations of VEGI. ^{3}H-Thymidine was added to the media prior to the mitotic phase. The amount of incorporated ^{3}H-thymidine was determined by scintillation counting (Black Bars). The number of cells (Striped Bars) was determined by cell counting. Panel B: Growth of G_0-synchronized ABAE cells as a function of time. The cells were seeded in triplicate in the absence (Blank Bars) or the presence of VEGI (60 ng/ml) (Striped Bars), and cell numbers determined at the indicated time points. Panel C: The growth inhibition is released. G_0-synchronized ABAE cells was re-seeded in the presence (open circles and closed triangles) or absence (closed circles) of VEGI (30 ng/ml). The media were replaced with fresh ones on day 3. VEGI was removed from the media of one the experimental groups (closed triangles) while that of the other experimental group continued to contain VEGI (open circles). The cultures were maintained for another 3 days. The number of cells in each well was counted at the indicated time intervals.

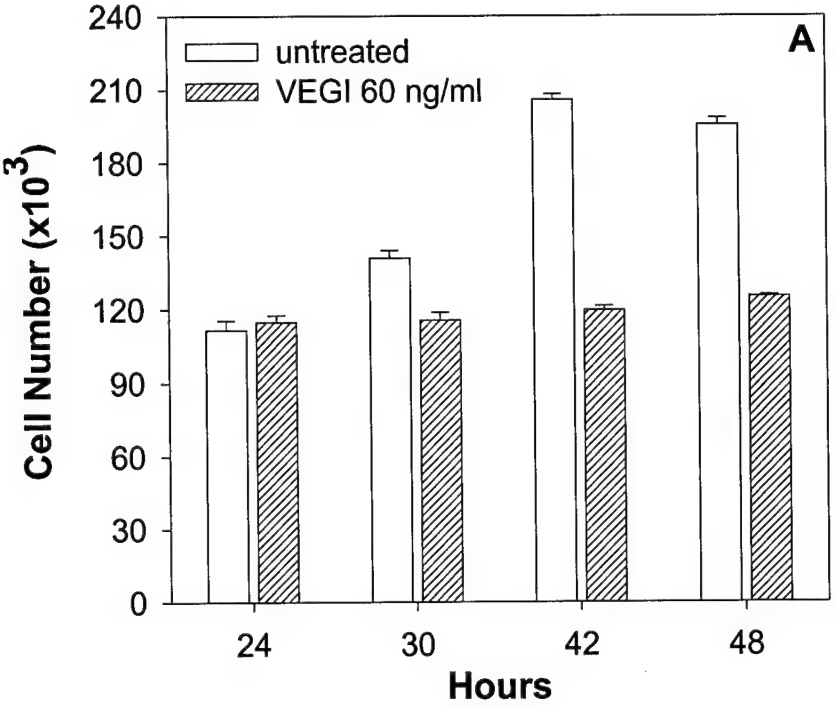
Figure 2. VEGI treatment of G_0-synchronized ABAE cells results in an early G_1 growth arrest as shown by Western blotting analysis of pRB and Myc. G_0-synchronized ABAE cells were seeded in the presence or absence of VEGI (60 ng/ml). The cells were harvested at the indicated time intervals. Total cell lysates were subjected to Western blotting analysis, using an monoclonal antibody to pRB, or to the Myc protein. The same membrane was analysed for β-actin as a protein loading control.

Figure 3. VEGI induced cell death of proliferating cells but not G_0 cells. VEGI-induced death of proliferating cells. G_0-synchronized ABAE cells were re-seeded. VEGI (60 ng/ml) was added at the indicated time (arrows). The culture media were replaced with fresh ones every two days. The cells were harvested in 24, 48, or 72 hours following each addition of VEGI, and the cell numbers determined.

Figure 4. FACS analysis of VEGI-induced apoptotic death to proliferating ABAE cells. Panel A: G_0 synchronized ABAE cells were seeded in the presence or absence of VEGI (60 ng/ml). VEGI was added to the culture media on Day 0 (at the time of seeding), Day 2, or Day 6. The cells were harvested in 24 hours following the addition of VEGI, and subjected to BrdU labeling of fragmented DNA and propidium iodine labeling of total DNA. FACS analysis for apoptosis was then carried out. The X-axis indicates the fluorescent intensity of BrdU-labeled cells, which is proportional to the extent of DNA fragmentation. The Y-axis is the fluorescent intensity of propidium iodine-incorporated cells, which indicates the distribution of the cells in the cell cycle, with diploid and tetraploid cells clustering at relative fluorescence intensity reading 50 and 100, respectively. Cells that appear in the upper right and the lower left quarters of the plots are

counted as apoptotic cells. Panel B: Plot of the percentage of apoptotic cells (A_0%) in the VEGI-treated cell populations (Striped Bars) versus untreated ones (Blank Bars). Indicated at the X-axis is the time when the cells were treated.

Figure 5: In-situ end-labeling (ISEL) analysis of VEGI-induced apoptosis of proliferating ABAE cells. G_0-synchronized ABAE cells were seeded in triplicate in the absence (A) or presence of VEGI (60 ng/ml), which was added to the culture media on Day 0 (B), Day 2 (C), or Day 6 (D). The cultures were maintained for 48 hours following each addition of VEGI. Incorporation of biotinylated UTP into fragmented nuclear DNA in the cells was analyzed by using ISEL. Purple staining (arrows) marks cells containing apoptotic bodies representing fragmented nuclear DNA.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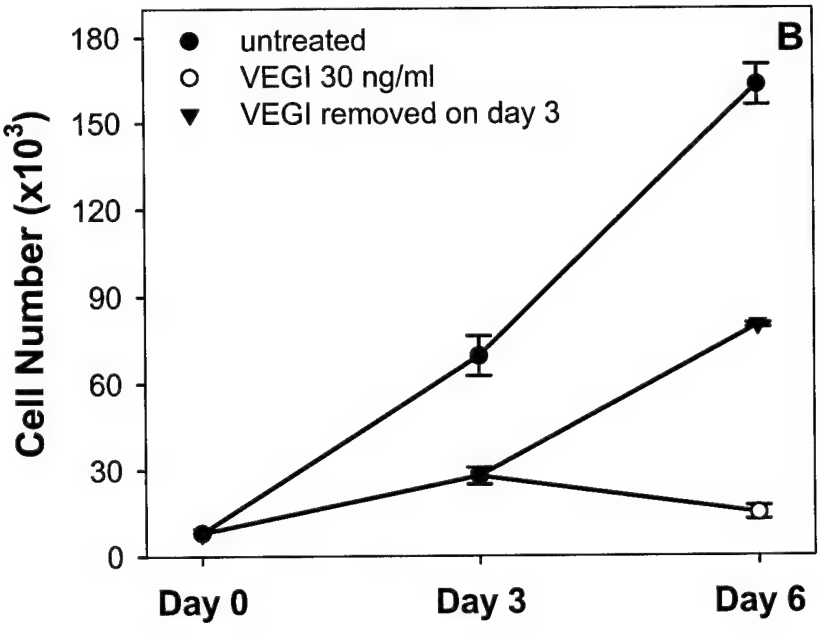
24
30
42
48
Hours


B
untreated
VEGI 30 ng/ml
VEGI removed on day 3
Cell Number (x10^3)
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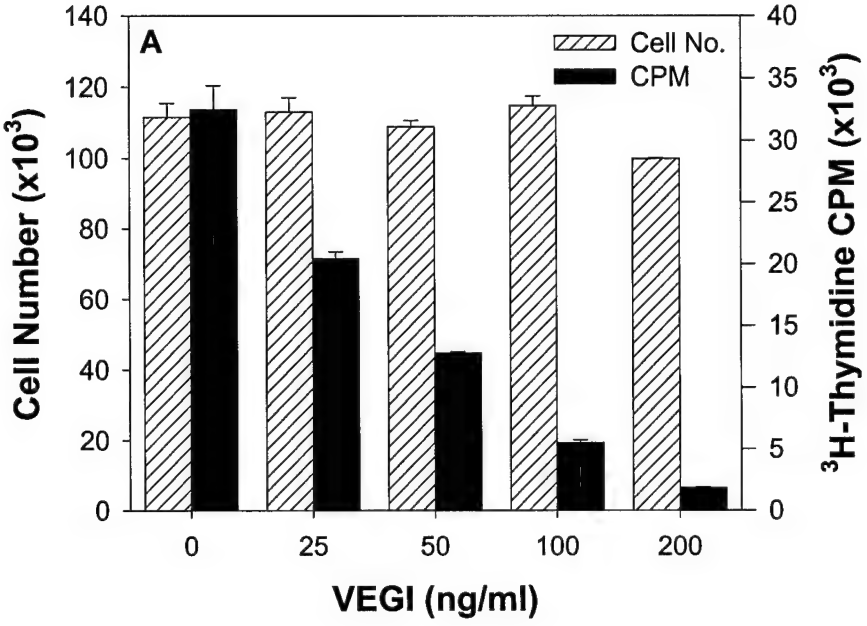
150
120
90
60
30
0
Day 0
Day 3
Day 6


A
Cell No.
CPM
Cell Number (x10^3)
^{3}H-Thymidine CPM (x10^3)
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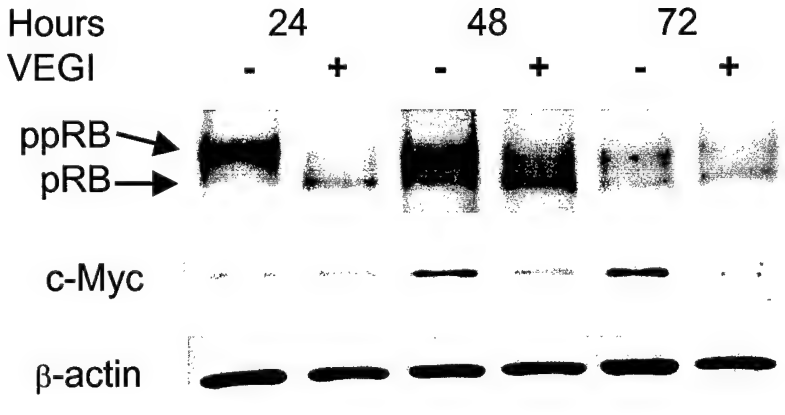
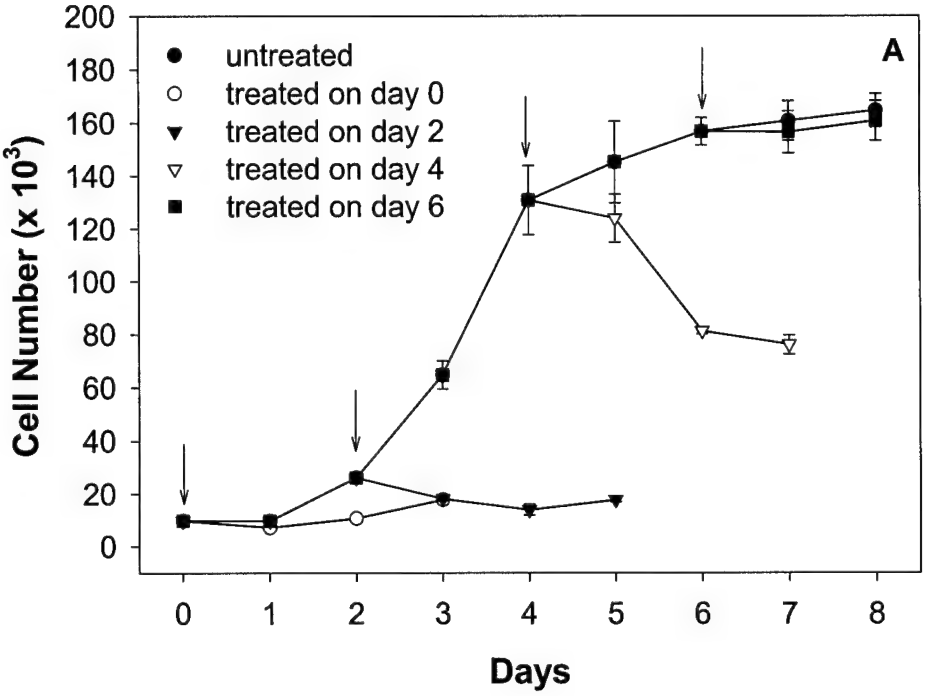
120
100
80
60
40
20
0
40
35
30
25
20
15
10
5
0
0
25
50
100
200
VEGI (ng/ml)

B


Hours
24
48
72
VEGI
- + - + - +
ppRB
pRB
c-Myc
β-actin


A
untreated
treated on day 0
treated on day 2
treated on day 4
treated on day 6
Cell Number (x 10^3)
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180
160
140
120
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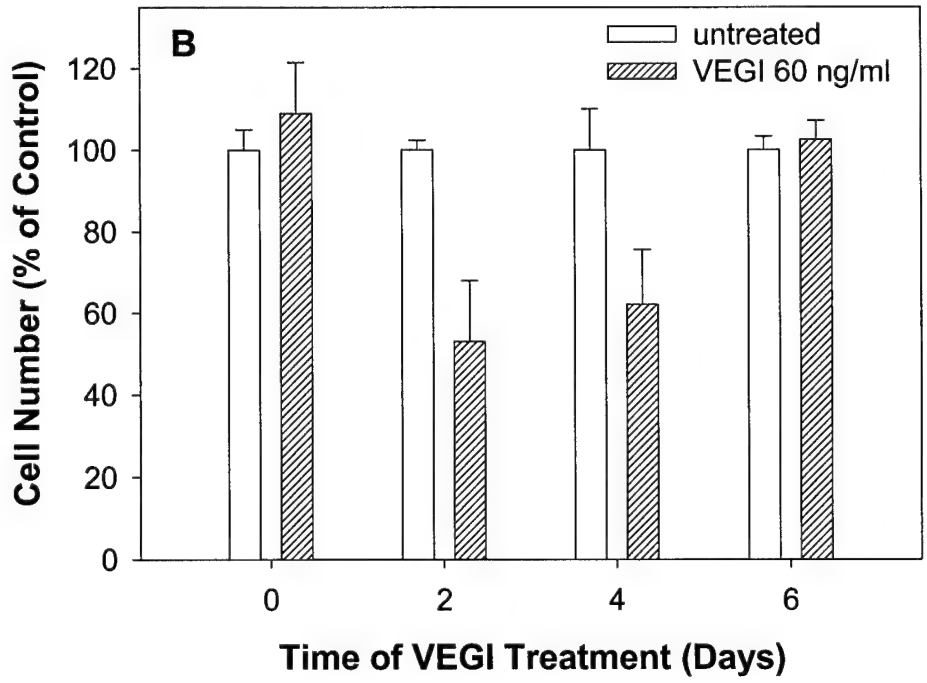
0
1
2
3
4
5
6
7
8
Days


B
untreated
VEGI 60 ng/ml
Cell Number (% of Control)
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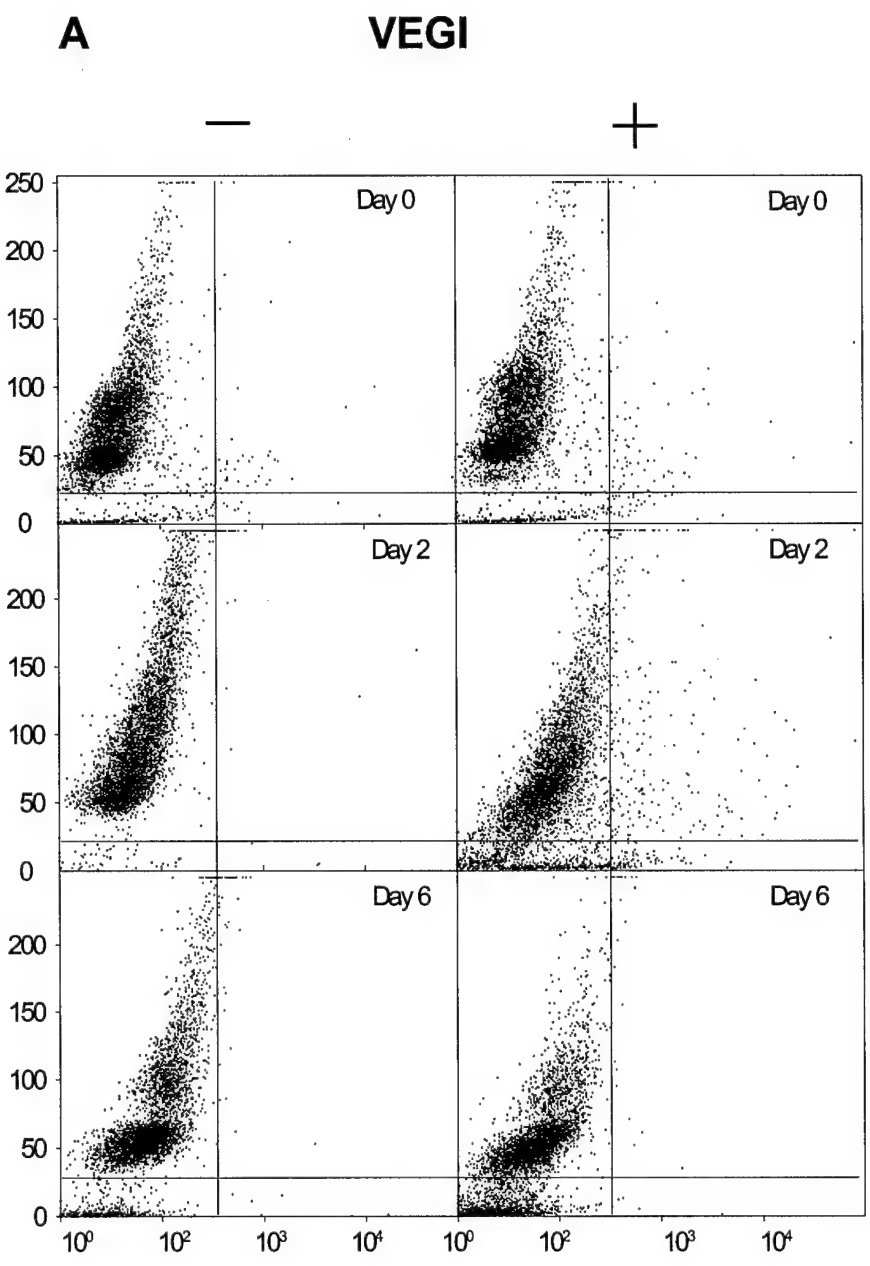
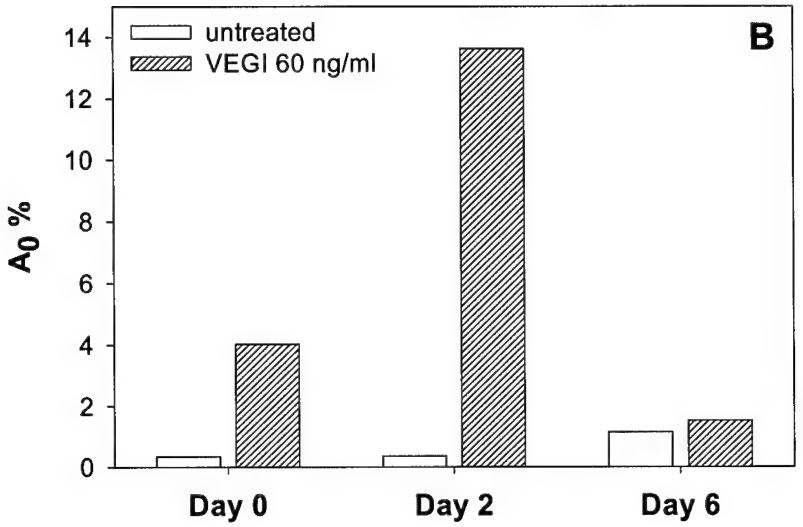
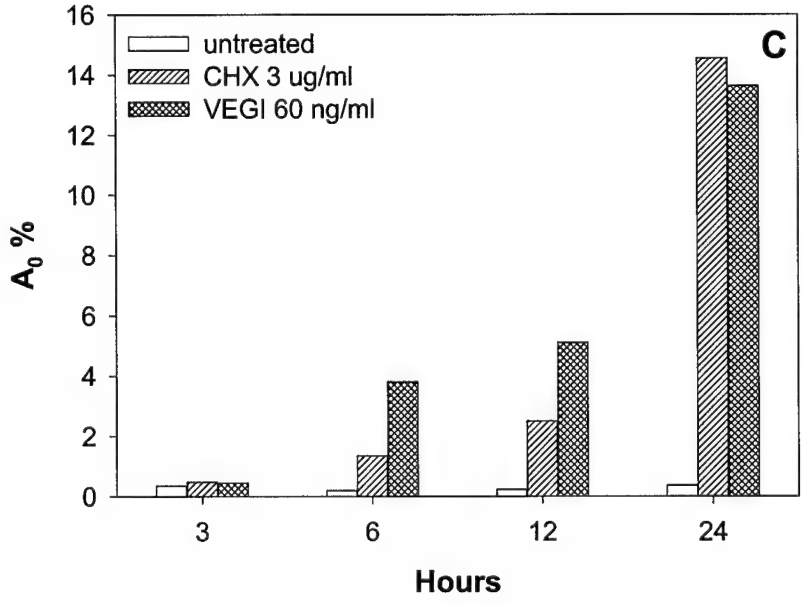
100
80
60
40
20
0
0
2
4
6
Time of VEGI Treatment (Days)

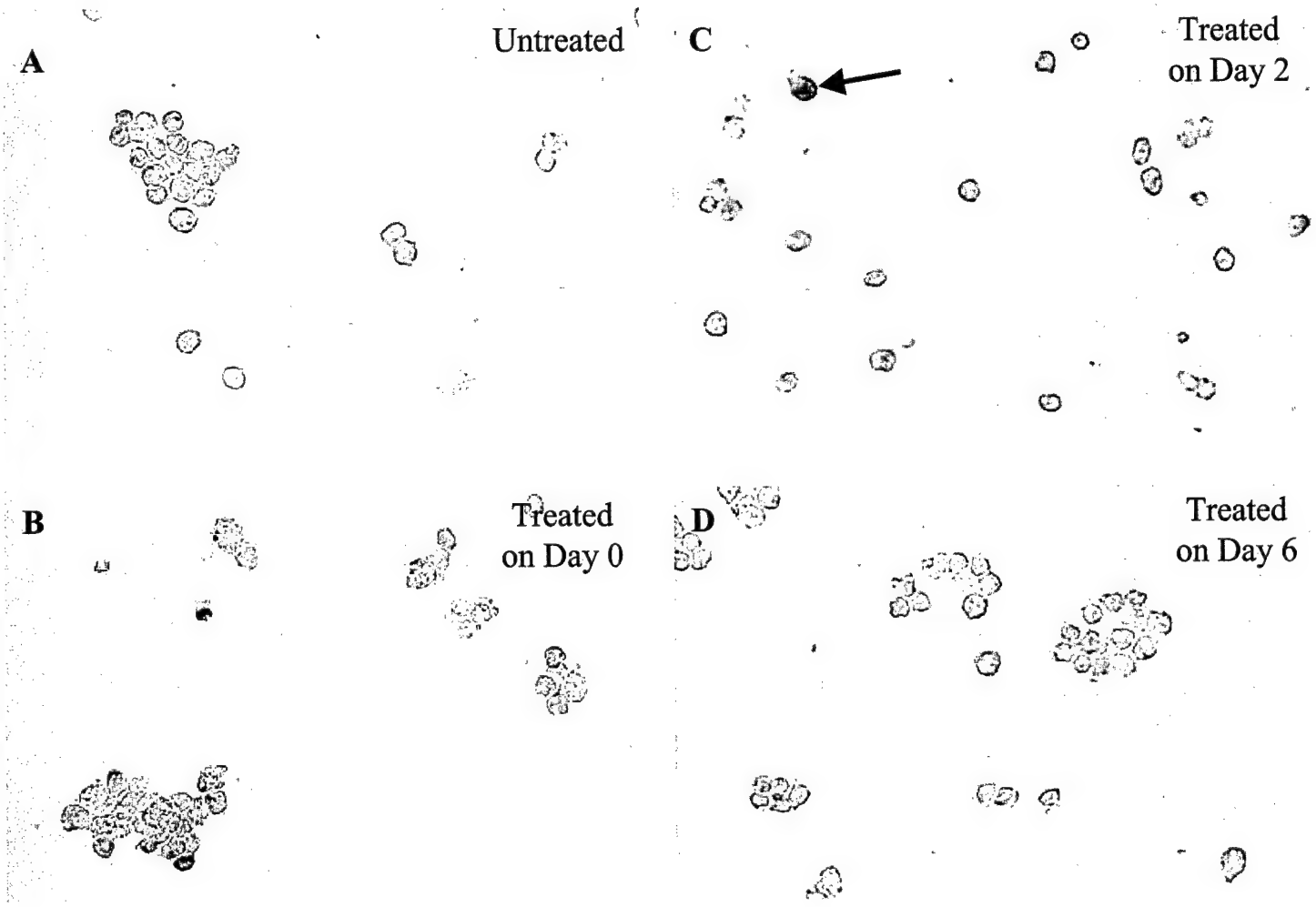

A
VEGI
—
+
Day 0
Day 0
Day 2
Day 2
Day 6
Day 6
250
200
150
100
50
0
10^0
10^2
10^3
10^4

Yu et al. Fig. 4BC





Yu etal Fig 5ABCD



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Int. J. Cancer: **82,** 131–136 (1999)

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INHIBITION OF ANGIOGENESIS AND BREAST CANCER XENOGRAFT TUMOR GROWTH BY VEGI, A NOVEL CYTOKINE OF THE TNF SUPERFAMILY

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Recently, we reported a novel protein of the tumor necrosis factor (TNF) superfamily, named vascular endothelial cell growth inhibitor (VEGI), which is expressed predominantly in endothelial cells. When a secreted form of this new protein was overexpressed in mouse colon cancer cells, the growth of tumors formed by these cells in black mice was inhibited. We now report that recombinant VEGI inhibits the proliferation of endothelial cells but not that of other types of cells examined. The protein also inhibits formation of capillary-like structures by endothelial cells in collagen gels, and the growth of capillaries into collagen gels placed on the chick chorioallantoic membrane. The anticancer potential of VEGI was examined in a breast cancer xenograft tumor model in which the cancer cells were co-injected with Chinese hamster ovary cells overexpressing a secreted form of the protein. The co-injection resulted in potent inhibition of xenograft tumor growth. Our findings are consistent with the view that VEGI is an endothelial cell-specific negative regulator of angiogenesis. ***Int. J. Cancer*** **82:131–136, 1999.**
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The endothelium plays an essential role in the maintenance of vascular homeostasis and permeability. Endothelial cells are actively involved in inflammation, cell adhesion, coagulation, thrombosis, fibrinolysis and angiogenesis. During angiogenesis, endothelial cells proliferate, invade into stroma and migrate toward the source of an angiogenic stimulus such as cancer cells. They then interact with perivascular cells and stromal cells to eventually form capillaries (Folkman, 1995). Although the mechanism of angiogenesis regulation is not fully understood, it is becoming clear that the initiation or termination of the process is controlled by a balance between positive and negative regulators of angiogenesis. A number of angiogenic factors have been described including several members of the fibroblast growth factor family such as FGF-1 (Gimenez-Gallego *et al.*, 1985), FGF-2 (Schweigerer *et al.*, 1987), those of the vascular endothelial cell growth factor family (VEGF) (Leung *et al.*, 1989), and the angiopoietins (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997). The receptors of these growth factors and cytokines have been identified (Burrus and Olwin, 1989; de Vries, 1992; Sato *et al.*, 1995; Terman *et al.*, 1992; Wennstrom *et al.*, 1991). Several inhibitors of angiogenesis have also been reported, including thrombospondin (Good *et al.*, 1990), angiostatin (O'Reilly *et al.*, 1994), endostatin (O'Reilly *et al.*, 1997) and platelet factor-4 (Maione *et al.*, 1990).

Physiological angiogenesis as is seen *in utero*, in wound healing or in the female reproductive tract is coordinated, being activated promptly when required and curtailed rapidly when further angiogenesis is no longer appropriate in that physiological setting. By contrast, pathological angiogenesis as seen in tumors, rheumatoid arthritis or diabetic retinopathy, once initiated is prolonged and ongoing with no termination despite the formation of new vessels. The failure to curtail pathological angiogenesis implies that a negative regulatory mechanism, which is functioning in physiological angiogenesis, is missing or suppressed in pathological angiogenesis. As both autocrine and paracrine negative feedback systems are common mechanisms by which proliferative physiological processes are curtailed, it is plausible that endothelial cells, which are a main component of the vasculature, may produce factors to suppress angiogenesis under physiological conditions. No such endothelial cell-produced negative regulator of angiogenesis has been described previously.

We have reported the identification of a novel cytokine, vascular endothelial cell growth inhibitor (VEGI) (Zhai *et al.*, 1999). The *VEGI* gene encodes a protein of 174 amino acids that exhibits a 20–30% overall sequence homology to the tumor necrosis factor superfamily. Among a wide variety of human cells examined, VEGI mRNA was found by Northern blotting analysis to be expressed only in endothelial cells. Total RNA obtained from many adult human organs and tissues also contains VEGI mRNA, suggesting its expression by endothelial cells of the quiescent vasculature. The sequence homology of VEGI to the tumor necrosis factor (TNF) family members prompted us to examine its function in a cancer model. The presence of a highly hydrophobic segment (residues 16–25) near the *N*-terminus of the protein suggests that VEGI is probably a type II membrane protein with a brief intracellular *N*-terminal segment and most of the protein (residues 26–174) being an extracellular domain, like most TNF family members (Aggarwal and Natarajan, 1996). Because many TNF family members are cleaved from the membrane to function as soluble proteins to affect distant target cells with appropriate receptors (Black *et al.*, 1997; Kayagaki *et al.*, 1995), we assumed a similar mechanism for VEGI. A secreted form of VEGI was, therefore, overexpressed in murine colon cancer cells (MC-38). The transfected cancer cells had greatly decreased tumorigenicity when implanted in C57/BL mice. Inhibition of tumor angiogenesis was evident from much decreased microvessel density. The conditioned media of the transfected cancer cells were found to be able to inhibit endothelial cell proliferation.

We have now made recombinant protein consisting of the putative extracellular domain of VEGI in *Escherichia coli* and examined its activity in a variety of cellular and animal models. The protein is able to inhibit the growth of endothelial cells but not that of other types of cells examined. The protein also inhibits formation of capillary-like structures by endothelial cells in collagen gels, and the growth of capillaries into collagen gels placed on the chick chorioallantoic membrane (CAM). We have overexpressed a secreted form of VEGI in Chinese hamster ovary (CHO) cells and co-injected the CHO cells with human breast cancer cells in nude mice so that VEGI protein would be made available at the tumor site. The co-injection resulted in marked inhibition of the growth of the breast cancer xenograft tumors.

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These findings suggest that VEGI is a negative regulator of angiogenesis produced predominantly by endothelial cells.

MATERIAL AND METHODS

Cell lines

NIH 3T3 cells, CHO cells, human breast cancer cell lines MDA-MB-231 and MDA-MB-435 were purchased from the American Type Culture Collections (ATCC, Rockville, MD). Human umbilical cord vein endothelial cells (HUVE) and human vascular smooth muscle cells (HVSM) were purchased from Clonetics (San Diego, CA). Adult bovine aortic endothelial cells (ABAE) were a gift from Dr. P. Bohlen (ImClone, New York).

VEGI recombinant protein preparation

A truncated form of VEGI consisting of residues 29–174 was fused with thioredoxin, which enhances the solubility of VEGI in *E. coli*, and a poly-histidine tag in a pET32a plasmid (Novagen, Madison, WI). The protein was purified from the soluble fraction of the homogenate of *E. coli* by affinity chromatography using Ni-Sepharose (Bio-Rad, Hercules, CA) to apparent homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The thioredoxin segment can be removed by using thrombin; however, the presence of thioredoxin in the fusion protein had no effect on the activity of VEGI. The fusion protein was, therefore, used as an equivalent of VEGI in the assays reported in these studies.

Proliferation assay

Cells were seeded in triplicate at 8,000 cells/well in 24-well plates, in IMEM (GIBCO, Gaithersburg, MD), 10% fetal calf serum (FCS) and cultured at 37°C, 5% CO_2. ABAE and HUVE cell culture media contained additional 1 ng/ml and 6 ng/ml FGF-2, respectively. The media were changed once on day 3. The number of viable cells was determined on day 6 by using a Coulter (Hialeah, FL) counter.

In vitro *angiogenesis assay*

Quantitative assessment of capillary-like tube formation by endothelial cells cultured on collagen gels were carried out as described elsewhere (Li *et al.*, 1994; Montesano and Orci, 1985). Three-dimensional collagen gel plates (24-well) were prepared by addition to each well of 0.5 ml chilled solution of 0.7 mg/ml of rat tail type I collagen (Becton Dickinson Franklin Lakes, NJ) in IMEM containing 10% fetal bovine serum (FBS) and adjusted to neutral pH with $NaHCO_3$. After formation of the collagen gel (about 1–2 mm thickness), ABAE cells were seeded at 50,000 cells/well. The cultures were maintained in IMEM, 10% FCS, 1 ng/ml of FGF-2, 5% CO_2, 37°C for 72 hr. The media were then replaced with fresh media omitting FGF-2 and cultured for 48 hr. The media were then replaced with fresh media containing 20 ng/ml of FGF-2. The cultures were maintained at 37°C for 48 hr. The gels were then fixed with cold methanol (−20°C). The abundance of the capillary-like structures formed by ABAE cells was determined by computer-assisted image analysis, using an IM35 inverted Zeiss microscope with phase contrast and Hoffman optics. Image acquisition and analysis is carried out using the Optimas 5.2 software (Media Cybernetics, Silver Spring, MD). The relative intensity of the capillary-like structures were measured as the ratio of the total length of the tubular structures over the total areas measured (mm/mm^2).

Chicken embryo CAM angiogenesis assay

Collagen gel pellets (0.05 ml) were placed on CAM. Angiogenesis in the gel pellet was induced by FGF-2 (50 ng) or VEGF (100 ng) supplemented in the gels. Various amount of VEGI was also incorporated into the gels. The extent of angiogenesis was determined by evaluation of the fluorescence intensity of FTIC-dextran injected into the CAM circulation prior to the retrieval of the gels and retained in the gels, as described previously (Iruela-Arispe and Dvorak, 1997).

Cancer cell-CHO cell co-inoculation in nude mice

A fusion protein consisting the secretion signal of interleukin-6 (Hirano *et al.*, 1986) and residues 23–174 of VEGI was constructed by PCR (5′ primer: 5′-GCGGGATCCG CCACCATGAA CTC-CTTCTCC ACAAGCGCCT TCGGTCCAGT TGCCTTCTCC CTGGGGCTGC TCCTGGTGTT GCCTGCTGCC TTCCCT-GCCC CAGTTGTGAG AC-3′). The primer contains a BamH I restriction endonuclease site (underlined), the first 84 bases of interleukin-6 coding sequence and 18 bases of VEGI starting from Pro23; 3′ primer: 5′-CGCGGATCCG ATATTTGCTC TCCT-CCTCA-3′, containing a BamH I restriction endonuclease site (underlined) and a stop codon and was inserted into an expression vector (pC1) that carries the dihydrofolate reductase gene. The plasmid was transfected into CHO cells that are dihydrofolate reductase negative. The stable CHO cell clones were selected for their ability to resist methotrexate, a dihydrofolate reductase inhibitor. The transfectants were maintained in modified Eagle's medium (MEM)-α containing 10% dialyzed FCS. CHO cell clones were further selected based on the presence of VEGI mRNA determined by Northern blotting analysis and the ability of the conditioned media, upon concentration, to inhibit ABAE cell growth. A quantitative assessment of VEGI protein in the conditioned media was not possible because of the lack of suitable antibodies. To ensure adequate production of VEGI at the tumor sites, 5×10^6 vector-transfected or secreted VEGI-overexpressing CHO cells were mixed with 10^6 human breast cancer cells (MDA-MB-231 or MDA-MB-435), an amount usually used in our inoculation of breast cancer cell lines. The cell mixtures were then injected into the mammary fat pads of female nude mice. Tumor sizes (mm^2) were measured in a blinded manner twice a week following injection.

RESULTS

Specific inhibition of endothelial cell proliferation by recombinant VEGI

A truncated form of VEGI consisting of residues 29–174 was expressed in *E. coli*. Among various truncations tested, the one reported here is the most active recombinant protein thus far. The protein was found to preferentially inhibit the FGF-2-induced proliferation of ABAE and HUVE while having no effect on the growth of HVSM, human breast cancer cells (MDAMB231) or NIH 3T3 cells (Fig. 1). The half-maximum inhibitory concentrations (IC_{50}) for ABAE and HUVE cells were about 6 ng/ml and 60 ng/ml, respectively. The difference in the IC_{50} values between the 2 cell types of different species probably reflects different affinities of the recombinant VEGI toward the receptors on the cell surface. The protein also had no effect on the proliferation of human T cells or bone marrow stromal cells at 100 ng/ml. These results suggest that VEGI, which is predominantly expressed by endothelial cells, specifically inhibits proliferation of endothelial cells when compared with other cell types tested.

Inhibition of in vitro *angiogenesis*

The anti-angiogenic activity of the recombinant VEGI was examined with an *in vitro* angiogenesis model. In this model, ABAE cells growing on a 3-dimensional collagen gel form a monolayer on the surface when the cell culture reaches confluence (Fig. 2*b*). Upon stimulation of the confluent monolayer cells with an angiogenic factor such as FGF2, however, many cells invade into the gel and form capillary-like tubular structures in the gel (Fig. 2*c*). When recombinant VEGI was added to the cell cultures together with FGF-2, inhibition of the formation of capillary-like tubes by ABAE cells was observed (Fig. 2*d*). The relative intensities of the tubules were determined by using computer-assisted image analysis. The IC_{50} value for the inhibition was found

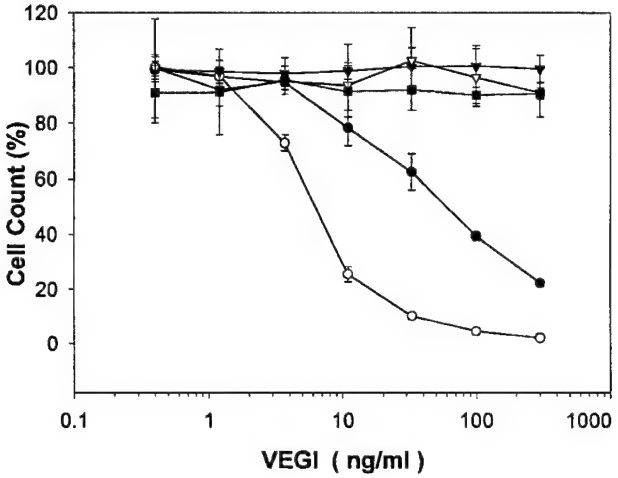

FIGURE 1 – Specific inhibition of endothelial cell proliferation by vascular endothelial cell growth inhibitor (VEGI). ●: human umbilical cord vein endothelial cells (HUVE); ○: adult bovine aortic endothelial cells (ABAE); ▼: human vascular smooth muscle cells (HVSM); ▽: human breast cancer cells (MDAMB231); ■: NIH3T3 mouse fibroblast cells. Cells were seeded in triplicate at 8,000 cells/well in 24-well plates. Culture conditions are described in Material and Methods. The media were changed once on day 3. The number of viable cells was determined on day 6 by using a Coulter counter. Mean values and standard deviations are presented as a function of VEGI concentrations.

to be approximately 30 ng/ml (Fig. 2*a*). Little cytotoxicity was observed on the confluent endothelial cells in the background monolayer (Fig. 2*d*). These results may have arisen from inhibition of proliferation and of certain differentiating functions involved in tubule formation.

Inhibition of chick CAM angiogenesis

The anti-angiogenic activity of VEGI was further determined by using a modified CAM assay. The method is based on the vertical growth of new capillary vessels into a collagen gel pellet placed on the CAM. The collagen gel was supplemented with an angiogenic factor such as FGF-2 (50 ng per gel or 1.0 μg/ml) or VEGF (100 ng per gel or 2.0 μg/ml), with or without VEGI. The high concentrations of the angiogenic factors were necessary to maintain a gradient in order to induce significant growth of capillaries into the gels. Because the size of the gel pellets (50 μl) was negligible, the angiogenic factors or inhibitors supplemented in the gels had no effect on the development of the CAM vasculature or that of the chick embryos. The extent of angiogenesis in the gel was assessed by using fluorescein isothiocyanate (FITC)-dextran, a fluorescent dye, injected into the circulation of the embryo. Simultaneously supplementing VEGI in the gels gave rise to a marked inhibition of the capillary growth in a dose-dependent manner. The concentration for VEGI to achieve half-maximum inhibition on FGF-2-induced angiogenesis was about 50 ng per gel (Fig. 3*a*). The half-maximum inhibitory concentration for VEGI to inhibit VEGF-induced angiogenesis was about 100 ng per gel (Fig. 3*b*). These IC_{50} values reflect a nearly one-to-one molar ratios between the stimulator and the inhibitor, because the m.w. of FGF-2, VEGF and VEGI are similar. These results indicate that the recombinant VEGI

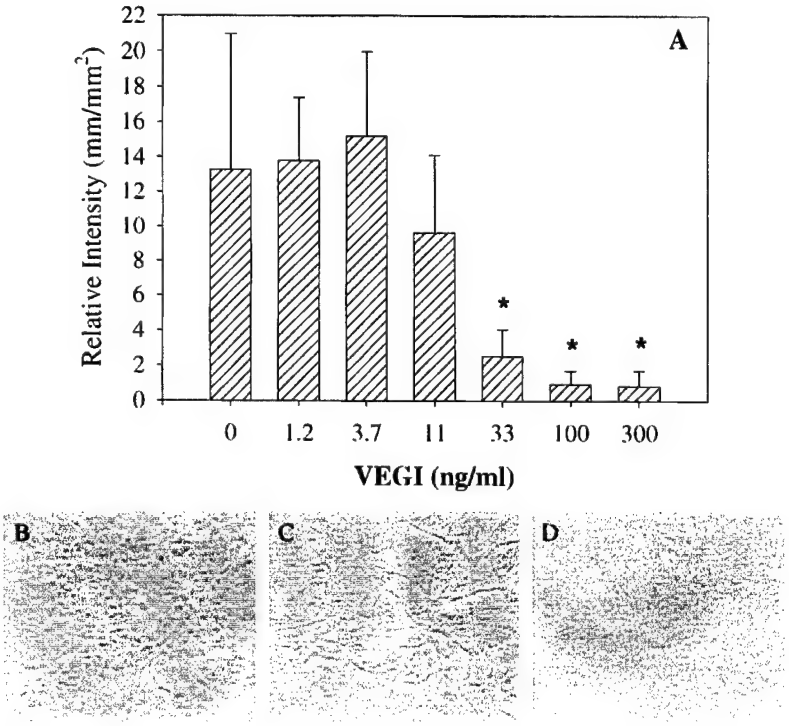

FIGURE 2 – Inhibition of formation of capillary-like tubules by adult bovine aortic endothelial cells (ABAE) cells in collagen gels. (*a*) Ability of vascular endothelial cell growth inhibitor (VEGI) to inhibit the formation of capillary-like tubes by ABAE cells is shown. The experiments were carried out in triplicate. At least 9 image areas were analyzed for each VEGI concentration. Mean values and standard deviations are presented as a function of VEGI concentration. Asterisks indicate $p < 0.05$ (ANOVA, Tukey test) by comparing to the tube intensity in the absence of VEGI. (*b*) ABAE cell monolayer on collagen gel. (*c*) Capillary-like tubes formed by ABAE cells when induced by fibroblast growth factor (FGF)-2 (50 ng/ml). Notice that the tube-like structures are on a different focal plane than the cell monolayer in the background. (*d*) Few tube-like structures are formed when VEGI (100 ng/ml) is present in the culture media. Notice the normal appearance of ABAE cells in the monolayer.

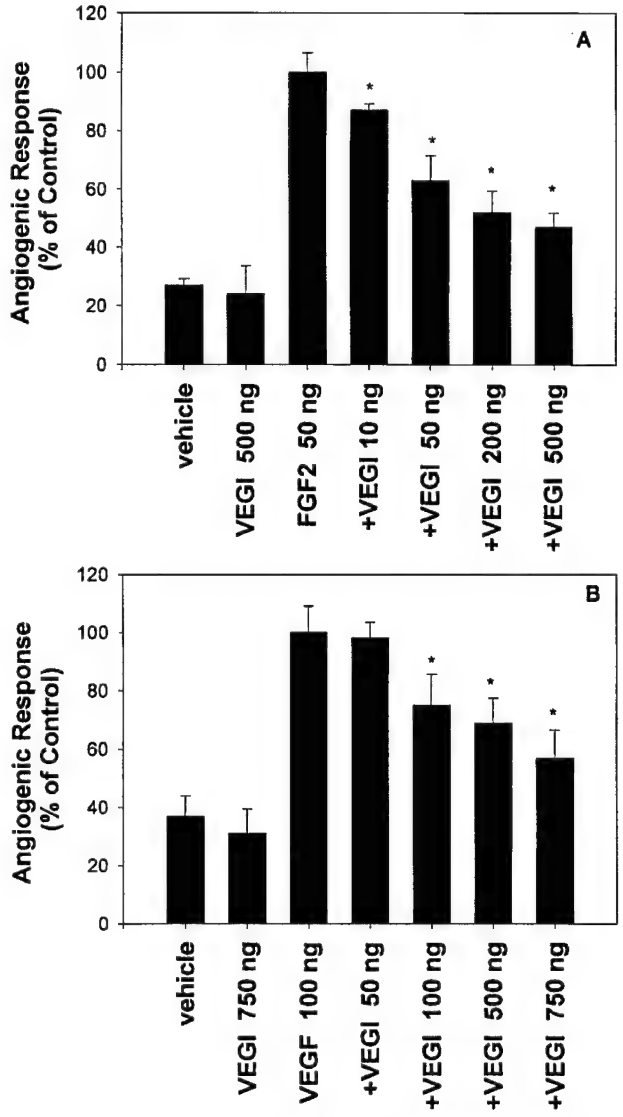

FIGURE 3 – Inhibition of chick embryonic chorioallantoic membrane (CAM) angiogenesis. (*a*) Inhibition of fibroblast growth factor (FGF)-2-induced angiogenesis in collagen gels placed on CAM. Angiogenesis in the gel pellet (0.05 ml) was induced by FGF-2 (50 ng) supplemented in the gels. Various amount of vascular endothelial cell growth inhibitor (VEGI), as indicated, was also incorporated into the gels. The extent of angiogenesis was determined by evaluation of the fluorescence intensity of FTIC-dextran injected into the CAM circulation before retrieval of the gels and retained in the gels. Error bars represent the standard deviation of quadruplicate experiments for each VEGI concentration. Asterisks indicate $p \leq 0.05$ (ANOVA, Tukey test) by comparing extent of FGF-2-induced angiogenesis. (*b*) Inhibition of vascular endothelial cell growth factor family (VEGF)-induced angiogenesis in the CAM under experimental conditions identical to described above, except that VEGF (100 ng) was used to induce angiogenesis.

was able to inhibit capillary growth regardless the cause of the angiogenic process.

Inhibition of human breast cancer xenograft tumor growth by CHO cells overexpressing VEGI

Because a negative regulator of angiogenesis would be expected to exert a inhibitory effect on tumor growth, the potential anticancer activity of VEGI was investigated, using breast cancer cells that are highly tumorigenic when implanted into the mammary fat pads of female athymic nude mice. Because VEGI would normally be found on endothelial cell membrane, perhaps in close juxtaposition to its receptor on the same cells, an *in vivo* experiment to test its anti-angiogenic properties might necessitate transduction of host endothelial cells with viral expression vectors. To avoid this experimentally difficult problem, and to deliver active VEGI to a tumor and surrounding stroma, a secreted form of VEGI was constructed by replacing the *N*-terminal segment containing the putative transmembrane of VEGI with a secretion signal peptide derived from human interleukin-6 (Hirano *et al.*, 1986). Because the secretion signal peptide is cleaved in the secretion vesicles, this strategy should give rise to the production of the same VEGI species used in the *in vitro* studies. The construct was transfected into CHO cells (clone 1.2). Expression of the corresponding mRNA was confirmed by Northern analysis. Secretion of the modified VEGI by the transfected cells was confirmed by the ability of the CHO cell-conditioned medium to inhibit ABAE cell growth when added to ABAE cultures, whereas no inhibitory activity was found in the conditioned media of full-length VEGI transfected cells (data not shown). This finding is consistent with our earlier finding that tumor cells transfected with full-length VEGI retained unaffected tumorigenicity (Zhai *et al.*, 1998), again applying that VEGI needs to be delivered to endothelial cells. The transfected CHO cells were mixed with human breast cancer cells (MDAMB231 or MDAMB435), and the cell mixtures were injected into the mammary fat pads of nude mice. A marked inhibition of the growth of the xenograft tumors formed by either the MDA-MB-231 (Fig. 4*a*) or the MDAMB435 cells (Fig. 4*b*) was observed. Vector-transfected CHO cells had no effect on tumor growth in either case. The experiments were repeated and similar results obtained. No tumor growth was observed when the transfected CHO cells were implanted alone (data not shown).

The experiment was repeated with another stable VEGI-overexpressing CHO cell line (clone 2.9). Interestingly, when MDA-MB-231 cells were co-injected with these CHO cells, there was an initial tumor growth in all animals; however, 5 of 6 animals showed marked decrease of the tumor sizes within 1–2 months (Fig. 4*c*). In sharp contrast, all animals had progressive tumor growth in the control group in which the cancer cells were co-injected with vector-transfected CHO cells (Fig. 4*d*). The experiments were repeated and similar results obtained. No tumor growth was observed when clone 2.9 cells were implanted alone (data not shown).

DISCUSSION

The activity of the recombinant VEGI was examined initially in a variety of biological assays, including the proliferation of different cell types. The only activity found to be associated with VEGI is the inhibition of endothelial cell proliferation. The potency of the inhibition was remarkable. Interestingly, no inhibitory effect was found on other types of cells examined under similar concentrations. This finding indicates that the target cells of VEGI are primarily endothelial cells. The ability of VEGI to suppress angiogenesis was illustrated with cellular and animal models. The formation of capillary-like tubules by endothelial cells in collagen gels is a process that exhibits many of the elemental endothelial cell activities during angiogenesis in addition to proliferation. The endothelial cells form a network of capillary-like tubular structures underneath a monolayer of confluent cells, which remains on the surface of the gel. The recombinant VEGI was able to prevent the tubule formation without apparent cytotoxic effect on the confluent cells. This result is in agreement with the fact that *VEGI* is expressed in many normal adult tissues, suggesting that this gene may be involved in suppressing the proliferation and differentiation of endothelial cells in a normally quiescent vasculature in adults. The results from the CAM model confirmed that VEGI can prevent neovascularization *in vivo*. In addition, inhibition of CAM angio-

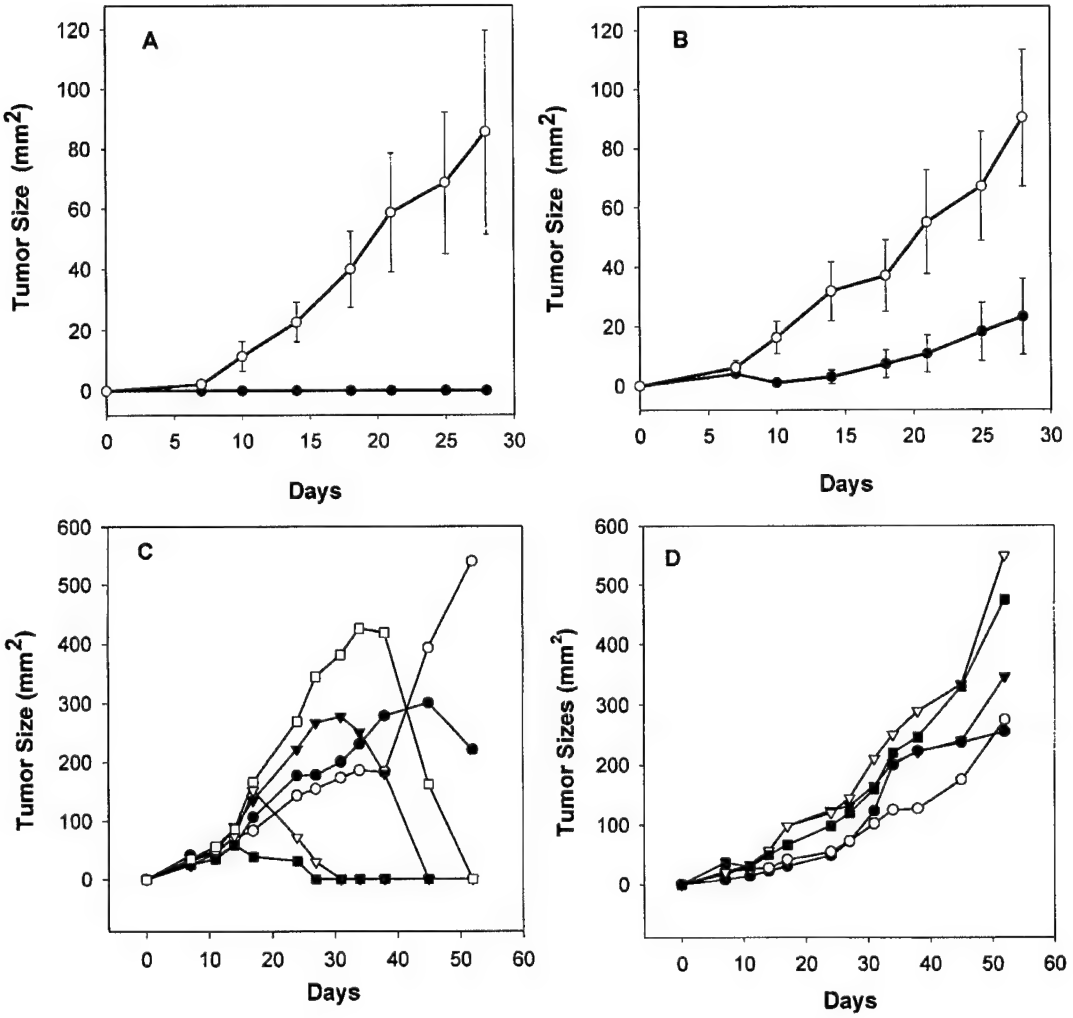

FIGURE 4 – Inhibition of the growth of 2 different human breast cancer xenograft tumors in nude mice by co-injected Chinese hamster ovary (CHO) cells overexpressing a secreted form of vascular endothelial cell growth inhibitor (VEGI). Vector-transfected or secreted VEGI-overexpressing CHO cells (5×10^6 (clone 1.2) were mixed with 10^6 cancer cells, then injected into the mammary fat pads of female nude mice (6 mice per group). Tumor sizes (mm^2) were monitored in a blinded manner. Mean values and standard deviations are presented as a function of days post-injection in (*a*) and (*b*). The experiments were repeated at least 2 times. Data shown were from one representative experiment. (*a*) Sizes of tumors formed by MDA-MB-231 cells co-injected with vector-transfected (○) or VEGI-producing CHO cells (●). (*b*) Sizes of tumors formed by MDA-MB-435 cells co-injected with vector-transfected (○) or VEGI-producing CHO cells (●). (*c*) Size of 6 individual tumors formed by MDA-MB-231 cells co-injected with VEGI-producing CHO cells (clone 2.9) in the experimental group. (*d*) Size of 5 individual tumors formed by MDA-MB-231 cells co-injected with vector-transfected CHO cells in the control group.

genesis by VEGI took place regardless what angiogenic factor induced the capillary growth. These results support the notion that VEGI is most likely not competing for the receptors of these very different growth factors, but exerting its activity by binding to a specific cell surface receptor to initiate a unique signaling pathway that would lead to the termination of angiogenesis.

The ideal method to demonstrate the potential anticancer effect of VEGI is a direct assessment of the recombinant protein in animal cancer models, but as yet we have been unable to prepare a sufficient amount of the protein to carry out this study. The CHO cell-cancer cell co-inoculation model was used as an alternative means of protein delivery. The results demonstrated that the release of VEGI into the vicinity of tumor cells leads to the inhibition of tumor growth. The lack of activity of full-length VEGI transfection of CHO cells or MC-38 mouse colon cancer cells also supports this conclusion. The striking anti-tumor effect of VEGI was likely to result from the ability of VEGI to suppress neovascularization, because recombinant VEGI had no inhibitory activity on the growth of the cancer cells *in vitro*, and secretion of VEGI by the transfected CHO cells had no inhibitory effect on their own growth. Furthermore, as shown by the collapse of the newly established xenograft tumors formed by the mixture of the cancer cells and the VEGI-overexpressing CHO cells, the action of VEGI probably led to eradication of tumor vasculature which is characterized by constant proliferation of endothelial cells. Similar eradication of newly established xenograft tumors was also observed when VEGI-overexpressing CHO cells (clone 1.2) were co-injected with prostate cancer PC3 cells. We demonstrated a dose-dependent tumor inhibition by altering the ratio of CHO:PC3 cells in the initial inoculum (data not shown). It is plausible that the amount of VEGI produced by co-injected CHO cells was initially insufficient to prevent the formation of the xenograft tumors; however, the CHO cells may grow together with the cancer cells in the tumors until a sufficient amount of VEGI was produced to effectively

terminate tumor neovascularization, which then led to the eradication of the tumors. Initially, the admixed tumor cells would provide the angiogenic stimulus for the growth of the CHO cells. It has been shown that CHO cells transfected with VEGF acquire the ability to proliferate dominantly in nude mice to form well-vascularized lesions, and the effect was attributed to the angiogenic activity of VEGF through paracrine mechanisms, because the CHO cells were not transformed (Ferrara *et al.*, 1993).

In conclusion, we presented evidence that VEGI, a novel cytokine predominantly expressed in endothelial cells, is a potent inhibitor of angiogenesis and tumor growth. Our findings are consistent with the view that VEGI may function as a negative regulator of angiogenesis involved in the maintenance of quiescence of normal vasculature or the termination of neovascularization. This is an example of an antiangiogenic factor produced by and acting on endothelial cells.

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REFERENCES

AGGARWAL, B.B. and NATARAJAN, K., Tumor necrosis factors: developments during the last decade. *Europ. Cytokine Netw.*, **7**, 93–124 (1996).

BLACK, R.A. and 19 OTHERS, A metalloproteinase disintegrin that releases tumor-necrosis factor-alpha from cells. *Nature (Lond.),* **385**, 729–733 (1997).

BURRUS, L.W. and OLWIN, B.B., Isolation of a receptor for acidic and basic fibroblast growth factor from embryonic chick. *J. biol. Chem.*, **264**, 18647–18653 (1989).

DAVIS, S., ALDRICH, T.H., JONES, P.F., ACHESON, A., COMPTON, D.L., JAIN, V., RYAN, T.E., BRUNO, J., RADZIEJEWSKI, C., MAISONPIERRE, P.C. and YANCOPOULOS, G.D., Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell,* **87**, 1161–1169 (1996).

DE VRIES, C., ESCOBEDO, J.A., UENO, H., HOUCK, K., FERRARA, N. and WILLIAMS, L.T., The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science,* **255**, 989–991 (1992).

FERRARA, N., WINER, J., BURTON, T., ROWLAND, A., SIEGEL, M., PHILLIPS, H.S., TERRELL, T., KELLER, G.A. and LEVINSON, A.D., Expression of vascular endothelial growth factor does not promote transformation but confers a growth advantage *in vivo* to Chinese hamster ovary cells. *J. clin. Invest.*, **91**, 160–170 (1993)

FOLKMAN, J., Angiogenesis in cancer, vascular rheumatoid and other diseases. *Nature (Med.)*, **1**, 27–31 (1995).

GIMENEZ-GALLEGO, G., RODKEY, J., BENNETT, C., RIOS-CANDELORE, M., DI SALVO, J. and THOMAS, K., Brain-derived acidic fibroblast growth factor: complete amino acid sequence and homologies. *Science,* **230**, 1385–1388 (1985).

GOOD, D.J., POLVERINI, P.J., RASTINEJAD, F., LE BEAU, M.M., LEMONS, R.S., FRAZIER, W.A. and BOUCK, N.P., A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc. nat. Acad. Sci. (Wash.),* **87**, 6624–6628 (1990).

HIRANO, T. and 15 OTHERS, Complementary DNA for a novel human interleukin (BSF2) that induces B lymphocytes to produce immunoglobulin. *Nature (Lond.),* **324**, 73–76 (1986).

IRUELA-ARISPE, M.L. and DVORAK, H.F., Angiogenesis: a dynamic balance of stimulators and inhibitors. *Thrombosis Haemostasis*, **78**, 672–677 (1997).

KAYAGAKI, N., KAWASAKI, A., EBATA, T., OHMOTO, H., IKEDA, S., INOUE, S., YOSHINO, K., OKUMURA, K. and YAGITA, H., Metalloproteinase-mediated release of human Fas ligand. *J. exp. Med.,* **182**, 1777–1783 (1995).

LEUNG, D.W., CACHIANES, G., KUANG, W.J., GOEDDEL, D.V. and FERRARA, N., Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science,* **246**, 1306–1309 (1989).

LI, L.Y., SAFRAN, M., AVIEZER, D., BOHLEN, P., SEDDON, A.P. and YAYON, A., Diminished heparin binding of a basic fibroblast growth factor mutant is associated with reduced receptor binding, mitogenesis, plasminogen activator induction, and *in vitro* angiogenesis. *Biochemistry,* **33**, 10999–11007 (1994).

MAIONE, T.E., GRAY, G.S., PETRO, J., HUNT, A.J., DONNER, A.L., BAUER, S.I., CARSON, H.F. and SHARPE, R., Inhibition of angiogenesis by recombinant human platelet factor4 and related peptides. *Science,* **247**, 77–79 (1990).

MAISONPIERRE, P.C. and 13 OTHERS, Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. *Science,* **277**, 55–60 (1997).

MONTESANO, R. and ORCI, L., Tumor-promoting phorbol esters induce angiogenesis *in vitro*. *Cell,* **42**, 469–477 (1985).

O'REILLY, M.S., BOEHM, T., SHING, Y., FUKAI, N., VASIOS, G., LANE, W.S., FLYNN, E., BIRKHEAD, J.R., OLSEN, B.R. and FOLKMAN, J., Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell,* **88**, 277285 (1997).

O'REILLY, M.S., HOLMGREN, L., SHING, Y., CHEN, C., ROSENTHAL, R.A., MOSES, M., LANE, W.S., CAO,Y., SAGE, E.H. and FOLKMAN, J., Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell,* **79**, 315–328 (1994).

SATO, T.N., TOZAWA, Y., DEUTSCH, U., WOLBURG-BUCHHOLZ, K., FUJIWARA, Y., GENDRON-MAGUIRE, M., GRIDLEY, T., WOLBURG, H., RISAU, W. and QIN, Y., Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature (Lond.),* **376**, 70–74 (1995).

SCHWEIGERER, L., NEUFELD, G., FRIEDMAN, J., ABRAHAM, J.A., FIDDES, J.C. and GOSPODAROWICZ, D., Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. *Nature (Lond.),* **325**, 257–259 (1987).

TERMAN, B.I., DOUGHER-VERMAZEN, M., CARRION, M.E., DIMITROV, D., ARMELLINO, D.C., GOSPODAROWICZ, D. and BOHLEN, P., Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem. biophys. Res. Comm.,* **187**, 1579–1586 (1992).

WENNSTROM, S., SANDSTROM, C. and CLAESSON-WELSH, L., cDNA cloning and expression of a human FGF receptor which binds acidic and basic FGF. *Growth Factors,* **4**, 197–208 (1991).

ZHAI Y. and 13 OTHERS, VEGI: a novel cytokine of the TNF family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas *in vivo*. *FASEB J.,* **13**, 181–189 (1999).

Modulation of Cell Cycle-Dependent Endothelial Cell Growth Arrest and Apoptosis by Vascular Endothelial Growth Inhibitor *

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ABBREVIATIONS: ABAE, adult bovine aortic endothelial cells; HUVE, human umbilical cord vein endothelial cells; pRB, the retinoblastoma gene product; VEGI, vascular endothelial growth inhibitor

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ABSTRACT

Vascular endothelial growth inhibitor (VEGI), an novel cytokine of the TNF-superfamily, is produced predominantly by endothelial cells and exhibits potent anti-angiogenic and anti-cancer activities (Zhai et al., Int. J. Cancer, 82:131, 1999). We report here that the effect of VEGI on endothelial cells is cell-cycle dependent: it mediates an early G_1 arrest in quiescent cells, but induces apoptotic death in proliferating cells. VEGI inhibits DNA synthesis in G_0-synchronized adult bovine aortic endothelial (ABAE) cells, which do not express VEGI themselves. The inhibition was reversible once VEGI was removed from the culture media. VEGI treated G_0-cells lacked typical markers of late G_1 phase, such as the hyperphosphorylation of the retinoblastoma gene product (pRB) and the upregulation of the *c-myc* gene, suggesting an early G_1 arrest. In contrast, exposure of ABAE cells that have entered the growth cycle to VEGI resulted in apoptotic cell death. Consistently, VEGI expression in human umbilical cord vein endothelial (HUVE) cells was found to be markedly upregulated in confluent cells as compared to that in proliferating cells. These findings support the view that, by modulating growth arrest and apoptosis of endothelial cells, VEGI participates in the maintenance of the quiescence of the endothelium of an established vasculature.

INTRODUCTION

The endothelium is the major component of the vascular system. It plays an important role in many vascular functions, including tissue homeostasis, fibrinolysis and coagulation, blood-tissue exchange, and neovascularization (1). The endothelium in an mature vasculature under physiological conditions is mostly a quiescent tissue (2). Maintenance of the quiescence of the endothelium is likely to involve the suppression of endothelial cell proliferation and the elimination of excessive endothelial cells. Endothelial cells become highly proliferative under certain conditions. The proliferation of endothelial cells in physiological angiogenesis as seen *in utero*, in wound healing, or in the female reproductive system, is apparently well-controlled by a balance between positive and negative regulators of angiogenesis (3,4). In contrast, pathological angiogenesis as seen in cancer, rheumatoid arthritis, and several other important diseases, is characterized by prolonged neovascularization with no termination. A negative regulation mechanism that functions in a physiological setting thus appears to be missing or poorly functioning in a pathological setting. A number of naturally existing inhibitors of angiogenesis have been reported. These include thrombospondin (5), platelet factor-4 (6), angiostatin (7), and endostatin (8). In addition, many growth factors and cytokines, such as platelet-derived growth factor (9), transforming growth factor-β (10), angiopoietin-1 (11), and angiopoietin-2 (12), have been shown to be involved in the stabilization and maturation of new blood vessels through modulations of endothelium-periendothelial cell interactions. However, the molecular mechanism underlying the maintenance of the extremely low turn over rate of the endothelial cells in a normal vasculature remains largely unclear.

We have recently reported the discovery of an endothelial cell-specific gene product, vascular endothelial cell growth inhibitor (VEGI) (13,14). The protein consists of 174-amino acids, with a 20-30% sequence homology to members of the TNF superfamily. Northern blotting analysis of a wide variety of cell lines and primary cell cultures indicates that the VEGI gene is expressed predominantly in endothelial cells. Additionally, the VEGI mRNA is detectable in many adult human organs, suggesting a physiological role of the gene in a normal vasculature. The function of VEGI was examined in a number of cellular and animal models. Recombinant VEGI inhibited endothelial cell proliferation with a remarkable potency, but had no effect on the growth of any other types of cells examined. The protein also inhibited the formation of capillary-like structures by endothelial cells in collagen gels, and the growth of capillaries into collagen gels placed on the chick chorioallantoic membrane. Overexpression of a secreted form of VEGI in murine colon cancer cells (MC-38) nearly completely prevented these cells to grow tumors in syngenic C57/BL mice. Moreover, co-inoculation of human breast cancer cells with Chinese hamster ovary cells overexpressing VEGI led to marked inhibition of the growth of the breast cancer xenograft tumors in nude mice.

We report here that the effect of VEGI on endothelial cells is cell-cycle dependent. Treatment of G_0-synchronized adult bovine aortic endothelial (ABAE) cells with VEGI gave rise to an early G_1 growth arrest, while exposure of proliferating ABAE cells to VEGI resulted in apoptotic cell death. In addition, VEGI expression in proliferating human umbilical cord vein endothelial (HUVE) cells was found to be markedly upregulated in confluent cells as compared to that in growing cells. Based on these findings, we propose that this cytokine may play a physiologically significant regulatory role as a suppressor of neovascularization in the maintenance of the quiescence of an established vasculature.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification: The primary sequence of VEGI contains 174 amino acid residues (GenBank Accession number AF039390). The recombinant VEGI consists of residues 29-174. The gene fragment was prepared from the cDNA template by PCR with a 5'-primer: 5'-CGCCATGGCC CATATGGCTC CCACACAGCAC-3' containing an Nco I restriction site and a 3'-primer: 5'-CCGGATCCTA TAGTAAGAAG GCTCC-3' containing a BamHI restriction site, then inserted into pET19b (Novegen, Madison, WI) to give a fusion protein that contains an N-terminal $(Histidine)_{10}$-tag. The fusion gene is expressed in E. coli (Strain BL21-DE3). Upon IPTG induction, the cells were homogenized by a repeated freeze-and-thaw process and intermittent sonication. The inclusion bodies were washed repeatedly with 25 mM HEPE-KOH buffer, pH 7.6, containing 10 mM EDTA, 100 mM NaCl, 1% Triton X-100 and 0.5 mM PMSF, then denatured in 50% acetic acid at room temperature for 2 hrs. Protein refolding was carried out at room temperature for 48 hrs in 25 mM HEPES-KOH, pH 7.6. The VEGI protein was purified by using affinity chromatography on an Ni-NTA agarose (Qiagen, Germany) column and 1 M immidazole elution. The molecular weight of the recombinant VEGI is estimated to be about 20 kDal by SDS-polyacrylamide gel electrophoresis (PAGE).

Cell synchronization and proliferation: Adult bovine aortic endothelial (ABAE) cells (a gift from Dr. Peter Bohlen of ImClone Inc., New York, NY) were cultured in IMEM (Gibco Biofluids, Rockville, MD), 10% FBS, 1 ng/ml fibroblast growth factor-2 (Promega, Madison, WI), 37 °C, 5% CO_2. The extent of quiescence of the cells was determined by ^{3}H-thymidine incorporation (see below). Cells were considered synchronized at G_0 phase of the cell cycle if no

more than 5% of the cells were incorporating ^{3}H-thymidine. The G_0-synchronized cells re-entered the growth cycle when they were re-seeded scarcely (5000 cells/cm^2) in IMEM with 10% FBS and 1 ng/ml fibroblast growth factor-2, and incubated at 37°C, 5% CO_2. Single cell suspension was prepared from each culture well at a given time interval by trypsinization. The number of cells in each suspension was determined by using a Coult Counter.

^{3}H-Thymidine incorporation: G_0-Synchronized ABAE cells were seeded at 1 x 10^5 cells/well in 12-well plates in the present of various concentrations of VEGI and incubated at 37°C, 5% CO_2 for 16 hrs. ^{3}H-Thymidine (Amersham, Piscataway, NJ) was then added (1 μCi/well) and the incubation continued for 6 hrs. The plates were then placed on ice. The cells were washed twice with ice-cold PBS, each time followed with a 5-min treatment with ice-cold trichloric acid. The cells were subjected to lysis in 0.5 ml NaOH (0.25 M) on ice for 30 min, and the lysates solutions subjected to scintillation counting.

Western blotting analysis: Cells were harvested by trypsinization, washed twice with PBS at 4°C, and collected by centrifugation at 4000 rpm for 4 min. The cells were resuspended in 25μl of cell lysis buffer (20mM Tris-HCI, pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 10% glycerol, 100 mM NaF, 200 μM Na_3VO_4, 4 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 10 μg/ml leupeptin) for 15 min on ice, then subjected to centrifugation at 13,000 rpm and 4°C for 15 min. The supernatant of the extracts was boiled in Laemmli sample buffer for 5 min and subjected to SDS-PAGE. The peptides on the PAGE gel were transferred onto an ECL-membrane (Amersham). After blocking with 5% skim milk in TBST buffer (20 mM Tris-HCI, pH7.5, 100 mM NaCl and 0.2 % Tween-20) for 1 hr, the membrane was incubated

overnight in 15 ml of TBST containing 5 μg/ml of a monoclonal antibody to human retinoblastoma protein (pRB) or to c-Myc (ZyMed, San Francisco, CA) at 4°C, washed with TBST, then incubated with goat anti-mouse HRP IgG (Pierce, Rockville, MD) for 1 hr, washed with TBST again, then with ECL reagent for 1 min. The membrane was then dried and subjected to autoradiography. The membrane was stripped and re-blotted with a monoclonal antibody to β-actin (ZyMed) for an internal control.

Flow cytometry analysis of apoptosis: Apoptotic cells were stained by using an APO-BrdU kit (Phoenix Flow Systems, San Diego, CA) which was based on the incorporation of bromodeoxyuridine (BrdU) into the 3'-hydroxyl group of fragmented DNA, as described (15). Briefly, ABAE cells (2×10^6) were harvested by trypsinization and suspended in PBS, then fixed with 10% paraformaldehyde for 15 minutes. The cells were collected by centrifugation, washed twice, treated with BrdU triphosphate, then with a fluorescein-conjugated anti-BrdU antibody. Propidium iodide was then used to stain total DNA, as described (16). The cell suspensions were analyzed by using a FACStar-Plus flow cytometry (Becton-Dickinson, Mountain View, CA).

In-situ end-labeling analysis of apoptosis: Cells were stained for apoptotic bodies using an in-situ end-labeling (ISEL) technique basically as described (17). ABAE cells were collected by trypsinization and centrifugation at 1000 rpm. The cell pellet were washed with ice cold PBS, then fixed in 10% paraformaldehyde for 10 min. The cells were centrifuged and washed in PBS repeatedly, resuspended in small aliquots of PBS, and placed on microscope slides. The slides were air-dried, re-hydrated with PBS for 10 minutes and then immersed in 0.3% hydrogen peroxide for 30 minutes. The slides were incubated at room temperature for 5 min in 50 mM

Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.001% 2-mercaptoethanosulphonic acid, and 0.005% BSA, then incubated at 37°C for 1 hr with the same buffer containing 0.2 mM each of dCTP, dATP and dTTP (Promega), 0.02 mM biotin-16-UTP (Boerhinger-Mannheim, Germany) and 20 U/ml Klenow DNA polymerase (Boerhinger-Mannheim). The slides were washed twice in PBS and incubated with an avidin-biotin-horseradish peroxidase conjugate prepared in PBS (Vectastain ABC, Vector Laboratories Burlinghame CA). The slides were then incubated with VIP substrate (Vector), counter-stained with methyl green (Vector), dehydrated and mounted with Permount (Fisher Scientific, Pittsburgh, PA), and subjected to microscopic examination.

Ribonuclease protection assay: Human umbilical cord vein endothelial (HUVE) cells (Clonetics, Walkersville, MD) were seeded in T-75 flasks (125,000 cells per flask) in IMEM, 10% FCS, 6 ng/ml FGF-2, 1 μg/ml heparin (Promega), 37°C, 5% CO_2. The culture media were replaced daily with fresh ones. Total RNA was prepared at the indicated time intervals. RNA probes for VEGI and 36B4 mRNA, a constantly expressed gene used as an internal control (18), radioactively labeled by using ^{32}P-UTP, were prepared by using a Ribonprobe Combination System kit, SP6/T7 (Promega). Total RNA (30 μg) from HUVE cells was mixed with the probes. Ribonuclease digestion was carried out by using a ribonuclease protection assay kit RPA-II (Ambion, Austin, TX). The mixtures were subjected to electrophoresis on 6% polyacrylamide gels, which were then dried and subjected to autoradiography. The intensities of the VEGI mRNA bands relative to the 36B4 bands were determined by using a PDI DNA-35 densitometer (PDI Inc., Huntington Station, NY). The relative intensity data were fitted to a simple exponential equation: $y = y_0 + a*\exp(b*x)$, $R = 0.99$. The cell number data were fitted to a sigmoidal regression equation: $y = y_0 + a/(1 + \exp(-b*(x - x_0)))$, $R = 0.999$.

RESULTS

VEGI-Treatment of G_0-Synchronized Endothelial Cells Causes a Reversible Growth Arrest: We investigated whether VEGI can suppress the initiation of endothelial cell proliferation in response to growth stimuli. ABAE cells were used in this study because these cells do not express VEGI but are highly responsive to this cytokine. The cells were synchronized in the G_0-phase of the cell cycle by cell-cell contact inhibition in confluent cultures. Once re-seeded in culture media, the cells re-entered the growth cycle and began to undergo the first mitotic division in about 20 hours following seeding. The cell number doubled once after about 40 hours (Fig. 1A). In the presence of VEGI (60 ng/ml), however, the cell number remained unchanged during the same period of time. The growth inhibition was reversible, as the cells resumed growth at a rate comparable to untreated cells once VEGI was removed from the culture media (Fig. 1B).

VEGI Induces Early G_1 Growth Arrest: In order to determine the phase of the cell cycle in which VEGI-induced growth arrest takes place, G_0-synchronized ABAE cells were seeded in the presence of various amount of VEGI, and their ability to carry out DNA synthesis was determined in a time interval prior to the first mitotic division. Cells treated with VEGI were unable to incorporate ^{3}H-thymidine (Fig. 2A). The inhibition was dose-dependent, with a half-maximum inhibition concentration (IC_{50}) of about 30 ng/ml (1.5 nM). More than 95% inhibition of ^{3}H-Thymidine incorporation was achieved at 200 ng/ml of VEGI. The number of cells remained basically unchanged under these conditions. It is thus apparent that VEGI-treated G_0-

synchronized endothelial cells were unable to advance to the S-phase in which DNA replication occurs.

We then determined the effect of VEGI treatment on the occurrence of typical markers of the G_1-phase of the cell cycle. A well-established marker for the late hours of the G_1 phase is the hyperphosphorylation of the retinoblastoma gene product, pRB, which is underphosphorylated in G_0 or early G_1 cells (19). To determine the phosphorylation status of the pRB protein, G_0-synchronized ABAE cells were seeded in the presence or absence of VEGI (15 ng/ml). The cells were harvested at various time intervals over a 3-day period, and subjected to Western blotting analysis, using a monoclonal antibody against pRB. The occurrence of a predominant, higher molecular weight species of pRB (Fig. 2B) indicated that the protein was mostly hyperphosphorylated within 24 hours following seeding in the absence of VEGI, whereas in VEGI treated cells the pRB protein was still mostly underphosphorylated, as represented by the lower molecular weight species (Fig. 2B), and remained as such as long as VEGI was present in the culture media. Thus, the growth arrest caused by VEGI treatment is highly likely to have taken place in the early G_1 phase. To further confirm this finding, we determined the expression of the *c-myc* gene, another well-studied late G_1 marker (20). It was clear that, determined by Western blotting analysis of the Myc protein, *c-myc* gene expression in ABAE cells following VEGI treatment was inhibited (Fig. 2B). The Myc protein level was elevated in the proliferating cells as expected, but was nearly depleted in G_0-synchronized ABAE cells cultured in the presence of VEGI for 72 hours. These data indicate that treatment of G_0-synchronized ABAE cells with VEGI gives rise to an early G_1 growth arrest.

VEGI Treatment Induces Cell Death of Proliferating ABAE Cells, But Not Quiescent Cells: We noticed during the course of the study that a variable number of cells were lost from the cultures following VEGI treatment, and realized that endothelial cells of different growing status responded to VEGI differently. We therefore compared the effect of VEGI on ABAE cells that have entered the growth cycle with that on quiescent cells. As mentioned earlier, G_0-synchronized ABAE cells upon re-seeding enter into the cell cycle and start the first mitotic division within about 20 hours. The cell number doubles approximately every 20 hours until the cell cultures are confluent once again in approximately 6 days, at which time the vast majority of the cells ceases to grow (Fig. 3A). VEGI was added to the cell cultures at various intervals during this period of time, namely, at the beginning when the G_0-cells were being re-seeded, in the middle of the logarithm growth phase of the cells in culture, and at the end when the cell cultures were nearly completely confluent (arrows, Fig. 3A). The cell numbers were determined in 24-hour intervals following each addition of VEGI. As expected, the cell numbers remained largely unchanged when the freshly seeded G_0-synchronized ABAE cells were treated with VEGI. No cell number change was observed in confluent cultures either. However, an marked loss of cells was found when the cultures were treated with VEGI on day 2 and day 4 following seeding, The cells at these time points were in the logarithm phase of growth. The cell number in these cultures decreased by as much as 50% and 40%, respectively, within 48 hours following the addition of VEGI to the culture media (Fig. 3B). These results indicate that, besides the induction of an early G_1 growth arrest of quiescent cells responding to growth stimuli, VEGI can also induce death of ABAE cells that have entered the cell cycle when they are exposed to this growth inhibitor.

Apoptosis Is the Cause of VEGI-Induced Death of Proliferating ABAE Cells: To determine whether programmed cell death was the cause of VEGI-induced death of the proliferating ABAE cells, the extent of nuclear DNA fragmentation in these cells was analyzed. G_0-synchronized ABAE cells were placed in culture. VEGI (60 ng/ml) was added to the media either at the time of seeding (day 0), on day 2, or on day 6. The cells were harvested in 48 hours following the addition of VEGI. The cells were treated with bromodeoxyuridine (BrdU) in order to label the 3'-hydroxyl ends of fragmented DNA to identify apoptotic cells. The cells were then subjected to fluorescent-activated cell sorting (FACS) (Fig. 4A). It was found that the percentage of the cells with extensive nuclear DNA fragmentation was significantly higher in cells treated with VEGI on day 2, when the cells were undergoing proliferation, as compared to that in cells treated either on day 0 at the time when the G_0-synchronized cells were being re-seeded, or on day 6 when the cell cultures were nearly completely confluent (Fig. 4A). The percentage of apoptotic cells in the cell cultures treated with VEGI on day 0, day 2, and day 6 were 5.5%, 14%, and 2%, respectively, when analyzed in 48 hours following the addition of VEGI to the culture media (Fig. 4B). A similar extent of apoptotic cell death was observed when ABAE cells were treated with cyclohexolamide, a common inducer of apoptotic death (Fig. 4C). Note that the VEGI concentration used in these experiments was about 2-times of the IC_{50} value. About 25% of the cells was thus expected to be able to grow in the presence of this amount of VEGI, as represented by a population of tetraploid cells in cultures treated with VEGI on day 0. This may account for the relatively higher percentage of apoptotic cells in cultures treated on day 0 as compared with that of untreated cells.

The VEGI-mediated cell cycle-dependent apoptosis was further investigated by using a different method. Again, G_0-synchronized ABAE cells were treated with VEGI on day 0, day 2,

and day 6. Fragmented DNA molecules in apoptotic cells were identified in 48 hours following VEGI treatment by using *in-situ* end-labeling with biotinylated UTP, then visualized with avidin-conjugated horseradish peroxidase. ABAE cells treated on day 2 displayed purple-colored discreet apoptotic bodies because of DNA fragmentation (Fig. 5C). The apoptotic bodies were rare in untreated cells, as well as in cells treated on day 0 and day 6 (Figs. 5A, 5B, 5D). The percentage of apoptotic cells in these populations was 2.3%, 12%, and 2.8%, respectively, for cells treated on day 0, day 2, and day 6, determined by counting the number of cells containing the apoptotic bodies (Fig. 5E). These data demonstrated that VEGI induces programmed cell death occurred only in endothelial cells that are undergoing proliferation.

Upregulation of VEGI Expression in Confluent HUVE Cells: VEGI was discovered initially from cDNA libraries prepared from HUVE cells (13). VEGI was also found to be a potent inhibitor of HUVE cell growth (14). We therefore investigated the relationship between the expression levels of this cytokine and the growth status of HUVE cells. G_0-synchronized HUVE cells were seeded under normal culture conditions, then harvested daily over a period of 7 days until the cell cultures were once again confluent (Fig. 6). The VEGI mRNA levels were determined by using ribonuclease protection assay. VEGI expression was found to be relatively low in proliferating HUVE cells, as seen in the first four days following seeding. When the cultures approached confluence, however, the VEGI mRNA levels increased exponentially, reaching a level on day 7 that is about 5-times of that seen with growing cells. The expression pattern of VEGI in HUVE cells thus is consistent with its function as a growth inhibitor of these cells.

DISCUSSION

Our data demonstrated that VEGI induces two distinct cellular activities in ABAE cells: suppression of the re-entry of G_0 cells into the cell growth cycle, or programmed death to cells that have already entered the cycle. Consistently, VEGI expression is significantly upregulated in confluent HUVE cells as compared to a relatively low level in proliferating cells. Previously we reported that VEGI is predominantly expressed in endothelial cells, that the VEGI mRNA is readily detectable in many human organs, and that VEGI apparently acts only endothelial cells (13,14). Together these data strongly support the view that VEGI is an endothelial cell-specific negative regulator of blood vessel growth. The physiological function of this unique cytokine may be to suppress the growth of endothelial cells in an mature vasculature, to terminate an undesirable neovascularization process, and to assist the removal of excessive endothelial cells from a regressing vasculature.

VEGI-induced growth arrest takes place in the early G_1-phase of the cell cycle. There are two distinguishable periods in the G_1 phase: early and late G_1. The entering of G_0 cells to early G_1 is reversible (21). Once the cells proceed to the late G_1 phase, they are obliged to continue into the S-phase. Since the VEGI treated G_0 cells were unable to incorporate ^{3}H-thymidine, the cells were apparently not able to proceed to the S-phase to synthesize DNA. We therefore analyzed the phosphorylation pattern of the pRB protein in ABAE cells in response to VEGI. The pRB protein is a key player in the decision-making process of a cell about growth or quiescence (19). The pRB protein undergoes a readily discernible chemical modification in a well-defined window of time. Through the preceding hours of in G_1, pRB is in an underphosphorylated form. During the last hours of G_1, most of the pRB molecules is

hyperphosphorylated. Our data clearly showed that the pRB protein did not undergo hyperphosphorylation in the presence of VEGI. These cells thus had not reached the late G_1-phase. It has been shown that the hyperphosphorylation of pRB leads to the inactivation of the growth inhibitory effect of this protein (22). This may be accounted for by the observation that the hyperphosphorylated pRB protein can no longer form a complex with the E2F family of transcription factors (23). The pRB-E2F complex actively suppresses the transcription of cell cycle genes. Moreover, pRB appears to be an integrator of both positive and negative signals. Factors that promotes cell proliferation should encourage pRB phosphorylation, whereas growth inhibitory signals, such as TGF-β and cell-cell contact, prevent pRB phosphorylation and thus block the progression of cell growth into late G_1 (24). VEGI may act as part of such inhibitory signals for endothelial cells.

Another G_1 marker is the Myc protein, a product of the early response gene c-*myc* whose transcription indicates the transition of the late G_1-phase to the S-phase (25). Myc is a positive regulator of G_1-specific cyclin-dependent kinase. The *c-myc* gene is activated by mitogenic signals, and is suppressed by growth inhibitory and differentiation signals. Additionally, the *c-myc* gene is subject to regulation by the pRB-E2F negative control mechanism (26). The binding of the pRB-E2F complex to the promoter of *c-myc* prevents transcription of the gene. Hyperphosphorylation of pRB leads to the dissociation of the pRB-E2F complex, which in turn permits the transcription of the target gene. Our data demonstrated that the *c-myc* gene expression is markedly upregulated in ABAE cells as they underwent the transition from G_0 to G_1 and subsequently entered the growth cycle. In the presence of VEGI, however, the Myc protein gradually diminished. This finding again indicates that VEGI prevents G_0-cells from advancing to late G_1-phase. In addition, the diminishing of Myc in VEGI treated G_0 cells is

consistent with the low apoptosis rate of these cells, since Myc is an essential player in the regulation of cell growth, differentiation, and death (20,27).

It is interesting that VEGI-induced apoptotic death of ABAE cells takes place only when these cells are undergoing proliferation. It has been reported previously that treatment of bovine pulmonary artery endothelial cells with a similar preparation of VEGI led to apoptosis (28). Cells at subconfluent densities were used in that study. Since the majority of cells in a subconfluent culture have not exited the cell cycle, the results are consistent with our findings. In the light that VEGI is expressed in the endothelium of many human organs, it is unlikely that this cytokine would induce apoptotic death to the quiescent cells in a normal endothelium. We found that no apoptosis was observed in G_0-synchronized ABAE cells treated with VEGI. The cytokine also did not cause apoptosis to G_0 cells in confluent cultures where the cell growth was inhibited by cell-cell contact. Additionally, the growth suppression of G_0-cells was reversed once VEGI was removed from the culture media. Only when the cells had entered the growth cycle, exposure to VEGI then gave rise to apoptotic cell death. These results strongly suggest that, in addition to functioning as a growth suppressor in a quiescent endothelium, VEGI may also function to terminate an angiogenesis process and remove excessive endothelial cells from a regressing vasculature, as seen in the cyclic processes of angiogenesis in wound healing, in the uterus during menstrual cycle, in the ovary during ovulation, as well as in the development of the embryonic cardiovascular system.

The primary sequence of VEGI contains a highly hydrophobic domain near the N-terminus (13). This suggests that VEGI, similar to most members of the TNF family (29), is a type-II membrane protein with an N-terminal segment located inside the cell while the rest of the protein forms an extracellular domain. The protein could function either as a membrane protein

or as a secreted factor if the extracellular domain is cleaved by proteolysis in a manner similar to the activation of a number of TNF-family members (30-32). Alternatively the N-terminal hydrophobic domain may serve as a secretion signal such that VEGI is produced by endothelial cells as a secreted cytokine. In either case, VEGI may serve as a negative regulator of endothelial cells in an autocrine mechanism in which the soluble cytokine binds to a receptor on endothelial cells, or in a juxtacrine mechanism through cell-cell contact. Similar mechanisms have been suggested to account for the action of the Fas ligand (33). It should be pointed out that a high level of VEGI in a quiescent endothelium is not necessarily an indication that the cytokine is playing an active role in that locality, since the presence of an appropriate receptor on the surface of these cells is pre-requisition for the system to be functional. Many members of the TNF receptor family have been described recently (34). It is likely that a candidate receptor for VEGI is specifically expressed, temporally or spatially, on endothelial cells, since this cell type appears to be the only target of this cytokine. It was reported recently that VEGI activated nuclear NF-κB in human histocytic lymphoma U-937 cells and inhibited the growth of several cancer cell lines in cell cultures (35). The concentrations of the VEGI preparation necessary for those activities to be observed, however, were about 10 μg/ml or higher, which are two to three orders of magnitude greater than what we and others (14,28) found to be needed for the inhibition of endothelial cells. It thus remains to be seen whether the inhibition of the growth of non-endothelial cells is a specific activity of VEGI.

The balance between proliferation and apoptosis of endothelial cells is likely to be the subject of a complicated regulatory mechanism. In addition to the activation of negative regulators and the down-regulation of positive regulators or their receptors, other factors such as signaling from the extracellular matrix acting through the integrins (36-38) and endothelium-

pericytes interactions (39), may also contribute to the regulation. The role of cytokines and growth factors in the suppression and termination of angiogenesis has only begun to be understood. Our findings provide new insights into a mechanism that potentially enables endothelial cells to negatively regulate their own proliferation and to promote programmed death. This mechanism could play an essential role in the maintenance of the quiescence of the endothelium of an established vasculature, in the termination of neovascularization, and in the regression of excessive blood vessels.

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REFERENCES

1. Risau, W. (1997) *Nature* **386**(6626), 671-4
2. Engerman, R. L., Pfaffenbach, D., and Davis, M. D. (1967) *Lab Invest* **17**(6), 738-43
3. Folkman, J. (1995) *Nat Med* **1**(1), 27-31
4. Hanahan, D., and Folkman, J. (1996) *Cell* **86**(3), 353-64
5. Good, D. J., Polverini, P. J., Rastinejad, F., Le Beau, M. M., Lemons, R. S., Frazier, W. A., and Bouck, N. P. (1990) *Proc Natl Acad Sci U S A* **87**(17), 6624-8
6. Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., Carson, H. F., and Sharpe, R. J. (1990) *Science* **247**(4938), 77-9
7. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. (1994) *Cell* **79**(2), 315-28
8. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. (1997) *Cell* **88**(2), 277-85
9. DiCorleto, P. E., and Bowen-Pope, D. F. (1983) *Proc Natl Acad Sci U S A* **80**(7), 1919-23
10. Antonelli-Orlidge, A., Saunders, K. B., Smith, S. R., and D'Amore, P. A. (1989) *Proc Natl Acad Sci U S A* **86**(12), 4544-8
11. Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1996) *Cell* **87**(7), 1171-80
12. Maisonpierre, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T. H., Papadopoulos, N., Daly, T. J., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1997) *Science* **277**(5322), 55-60

13. Zhai, Y., Ni, J., Jiang, G., Lu, J., Xing, L., Lincoln, C., Carter, K. C., Janat, F., Kozak, D., Xu, S., Rojas, L., Aggarwal, B. B., Ruben, S., Li, L., Gentz, R., and Yu, G. (1999) *Faseb J* **13**(1), 181-9

14. Zhai, Y., Yu, J., Iruela-Arispe, L., Huang, W., Wang, Z., Hayes, A., Lu, J., Jiang, G.W., Rojas, L., Lippman, M.E., Ni, J., Yu, G.L., Li, L.Y. (1999) *Int. J. Cancer* **82,** 131-6

15. Dolbeare, F., Gratzner, H., Pallavicini, M. G., and Gray, J. W. (1983) *Proc Natl Acad Sci U S A* **80**(18), 5573-7

16. Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M. A., Lassota, P., and Traganos, F. (1992) *Cytometry* **13**(8), 795-808

17. Wijsman, J. H., Jonker, R. R., Keijzer, R., van de Velde, C. J., Cornelisse, C. J., and van Dierendonck, J. H. (1993) *J Histochem Cytochem* **41**(1), 7-12

18. Laborda, J. (1991) *Nucleic Acids Res* **19**(14), 3998

19. Weinberg, R. A. (1995) *Cell* **81**(3), 323-30

20. Amati, B., Alevizopoulos, K., and Vlach, J. (1998) *Front Biosci* **3,** D250-68

21. Pardee, A. B. (1989) *Science* **246**(4930), 603-8

22. Cobrinik, D., Dowdy, S. F., Hinds, P. W., Mittnacht, S., and Weinberg, R. A. (1992) *Trends Biochem Sci* **17**(8), 312-5

23. Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. (1991) *Cell* **65**(6), 1053-61

24. Wang, J. Y., Knudsen, E. S., and Welch, P. J. (1994) *Adv Cancer Res* **64,** 25-85

25. Marcu, K. B., Bossone, S. A., and Patel, A. J. (1992) *Annu Rev Biochem* **61,** 809-60

26. Oswald, F., Lovec, H., Moroy, T., and Lipp, M. (1994) *Oncogene* **9**(7), 2029-36

27. Henriksson, M., and Luscher, B. (1996) *Adv Cancer Res* **68,** 109-82

28. Yue, T. L., Ni, J., Romanic, A. M., Gu, J. L., Keller, P., Wang, C., Kumar, S., Yu, G. L., Hart, T. K., Wang, X., Xia, Z., DeWolf, W. E., Jr., and Feuerstein, G. Z. (1999) *J Biol Chem* **274**(3), 1479-86

29. Aggarwal, B. B., and Natarajan, K. (1996) *Eur Cytokine Netw* **7**(2), 93-124

30. Kayagaki, N., Kawasaki, A., Ebata, T., Ohmoto, H., Ikeda, S., Inoue, S., Yoshino, K., Okumura, K., and Yagita, H. (1995) *J Exp Med* **182**(6), 1777-83

31. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) *Nature* **385**(6618), 729-33

32. Nagata, S. (1997) *Cell* **88**(3), 355-65

33. Giordano, C., Stassi, G., De Maria, R., Todaro, M., Richiusa, P., Papoff, G., Ruberti, G., Bagnasco, M., Testi, R., and Galluzzo, A. (1997) *Science* **275**(5302), 960-3

34. Ashkenazi, A., and Dixit, V. M. (1999) *Curr Opin Cell Biol* **11**(2), 255-60

35. Haridas, V., Shrivastava, A., Su, J., Yu, G. L., Ni, J., Liu, D., Chen, S. F., Ni, Y., Ruben, S. M., Gentz, R., and Aggarwal, B. B. (1999) *Oncogene* **18**(47), 6496-504

36. George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H., and Hynes, R. O. (1993) *Development* **119**(4), 1079-91

37. Brooks, P. C., Clark, R. A., and Cheresh, D. A. (1994) *Science* **264**(5158), 569-71

38. Bader, B. L., Rayburn, H., Crowley, D., and Hynes, R. O. (1998) *Cell* **95**(4), 507-19

39. Hirschi, K. K., and D'Amore, P. A. (1996) *Cardiovasc Res* **32**(4), 687-98

FIGURE LEGEND

Figure 1. Growth inhibition of ABAE cells by VEGI. Panel A: Growth of G_0-synchronized ABAE cells as a function of time. The cells were seeded at 1 x 10^4 per well in 24-well plates, in triplicate, in the absence (Blank Bars) or the presence of VEGI (60 ng/ml) (Striped Bars), then harvested at the indicated time points and counted. Panel B: Growth inhibition is released once VEGI is removed from culture media. G_0-synchronized ABAE cells was re-seeded as above in the presence (open circles and closed triangles) or absence (closed circles) of VEGI (30 ng/ml). The media were replaced with fresh ones on day 3. VEGI was removed from the media of one the experimental groups (closed triangles) while that of the other experimental group continued to contain VEGI (open circles). The cultures were maintained for another 3 days. The number of cells in each well was counted at the indicated time intervals.

Figure 2. VEGI treatment of G_0-synchronized ABAE cells results in an early G_1 growth arrest. Panel A: Inhibition of ^{3}H-thymidine incorporation. G_0-synchronized ABAE cells were seeded in 24-well plates at 1 x 10^4 cells per well, in triplicate, in the presence of indicated concentrations of VEGI and cultured for 16 hours. ^{3}H-Thymidine was added to the cultures. The cells cultures were maintained for an additional 6 hours prior to harvest. The amount of incorporated ^{3}H-thymidine was determined by scintillation counting (Black Bars). The number of cells (Striped Bars) was determined by cell counting. Panel B: Western blotting analysis of pRB and Myc. G_0-synchronized ABAE cells were seeded in the presence or absence of VEGI (15 ng/ml). The cells were harvested at the indicated time intervals. Total cell lysates were subjected to Western

blotting analysis, using an monoclonal antibody to pRB, or to the Myc protein. The same membrane was analysed for β-actin as a protein loading control.

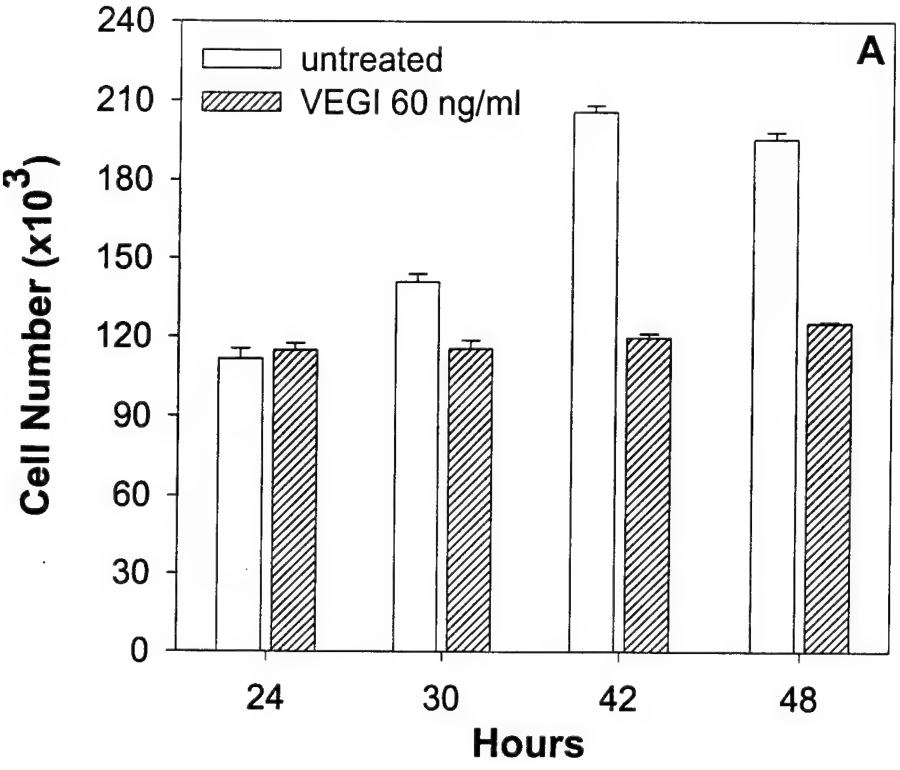
Figure 3. VEGI induced growth arrest of G_0 cells but cell death of proliferating cells. Panel A: VEGI-induced death of proliferating cells. G_0-synchronized ABAE cells were seeded in 24-well plates at 1 x 10^4 cells per well. VEGI (60 ng/ml) was added at the indicated time (arrows). The culture media were replaced with fresh ones every two days. The cells were harvested in 24, 48, or 72 hours following the addition of VEGI, and the cell numbers determined. Panel B: Comparison of loss of cells in cultures in the absence (Blank Bars) or presence of VEGI (Striped Bars) added to the culture media at various time points. The cell numbers were determined in 48 hours following the addition of VEGI at the indicated time intervals, and compared to those of the untreated cultures determined two days earlier at the time when VEGI was added to the treated groups.

Figure 4. FACS analysis of VEGI-induced apoptotic death to proliferating ABAE cells. Panel A: G_0 synchronized ABAE cells were seeded in the presence or absence of VEGI (60 ng/ml). VEGI was added to the culture media on Day 0 (at the time of seeding), Day 2, or Day 6. The cells were harvested in 24 hours following the addition of VEGI, and subjected to BrdU labeling of fragmented DNA and propidium iodine labeling of total DNA. FACS analysis for apoptosis was then carried out. The X-axis indicates the fluorescent intensity of BrdU-labeled cells, which is proportional to the extent of DNA fragmentation. The Y-axis is the fluorescent intensity of propidium iodine-incorporated cells, which indicates the distribution of the cells in the cell cycle, with diploid and tetraploid cells clustering at relative fluorescence intensity reading 50 and 100,

respectively. Apoptotic cells appear in the upper right quarter of the plot. Panel B: Plot of the percentage of apoptotic cells (A_0%) in the VEGI-treated cell populations (Striped Bars) versus untreated ones (Blank Bars). Indicated at the X-axis is the time when the cells were treated. Panel C: Comparison of VEGI and cyclohexolamide induced endothelial cell apoptosis. ABAE cell cultures were either treated with cyclohexolamide (3 μg/ml) (Striped Bars) or VEGI (60 ng/ml) (Crossed Bars), or untreated (Blank Bars). The percentage of apoptotic cells was determined by FACS analysis at the indicated time intervals.

Figure 5: In-situ end-labeling (ISEL) analysis of VEGI-induced apoptosis of proliferating ABAE cells. G_0-synchronized ABAE cells were seeded in triplicate in the presence or absence of VEGI (60 ng/ml), which was added to the culture media on Day 0 (at the time of seeding), Day 2, or Day 6. The cultures were maintained for 48 hours following each addition of VEGI. Incorporation of biotinylated UTP into fragmented nuclear DNA in the cells was analyzed by using ISEL. Panels A-D: Microscopic photographs showing typical appearance of untreated or treated cells at the indicated time. Purple staining (arrow) marks cells containing apoptotic bodies representing fragmented nuclear DNA. Panel E: Percentage of ISEL-positive cells was quantitatively determined by counting purple-colored cells under a light microscope. Four randomly selected fields on each slide and three slides for each experiment were analyzed. The total number of cells and that of ISEL-positive cells in each field were counted. The percentage of apoptotic cells in VEGI-treated cultures (Striped Bars) was compared to that of untreated cultures (Blank Bars).

Figure 6. Upregulation of VEGI expression in HUVE cells. Panel A: Autoradiography of VEGI mRNA analyzed by using ribonuclease protection assay. G_0-synchronized HUVE cells were seeded in at a density of 2000 cells/cm^2 in T-75 flasks. The culture media were replaced daily with fresh ones. The cells were harvested and counted at the indicated time intervals. Total RNA was prepared and subjected to ribonuclease protection assay. Panel B: Semi-quantitative analysis of VEGI mRNA levels based on the ribonuclease protection assay. The intensity of the VEGI (triangles) was determined by using a PDI-densitometer and normalized based on the intensity of the 36B4 mRNA control bands on the same lane. The number of cells per flask (circles) was determined by using a Coult Counter.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untreated
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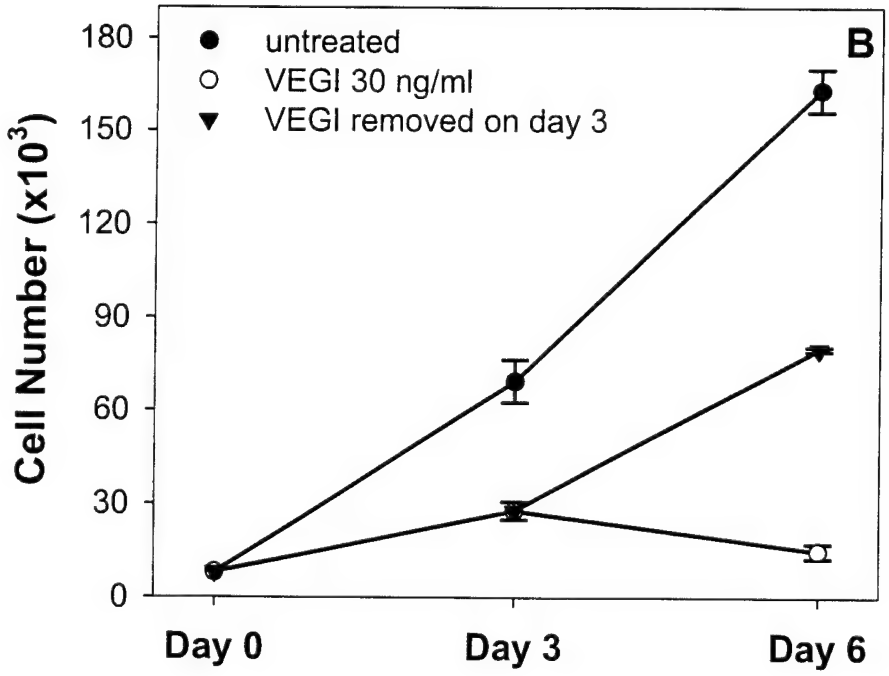
180
150
120
90
60
30
0
24
30
42
48
Hours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VEGI 30 ng/ml
VEGI removed on day 3
Cell Number (x10^3)
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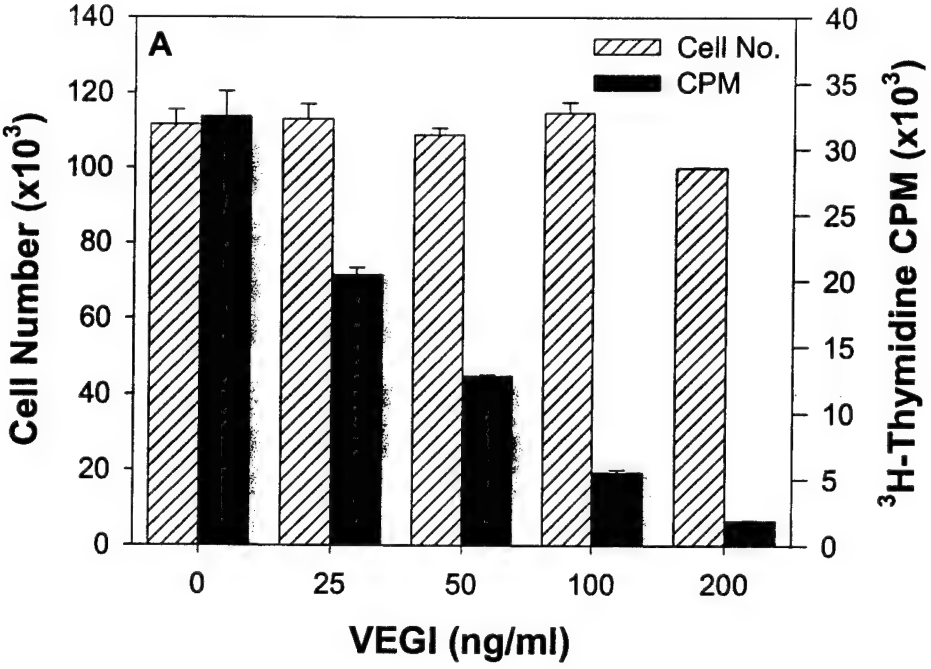
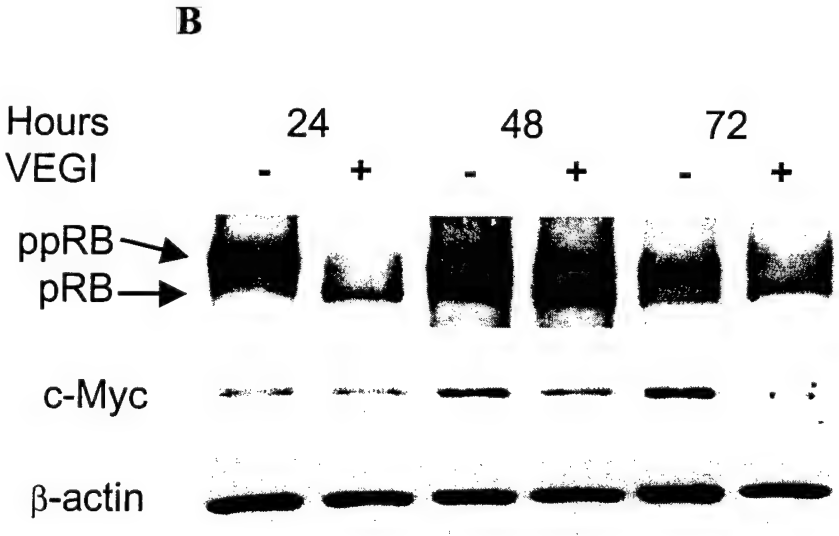
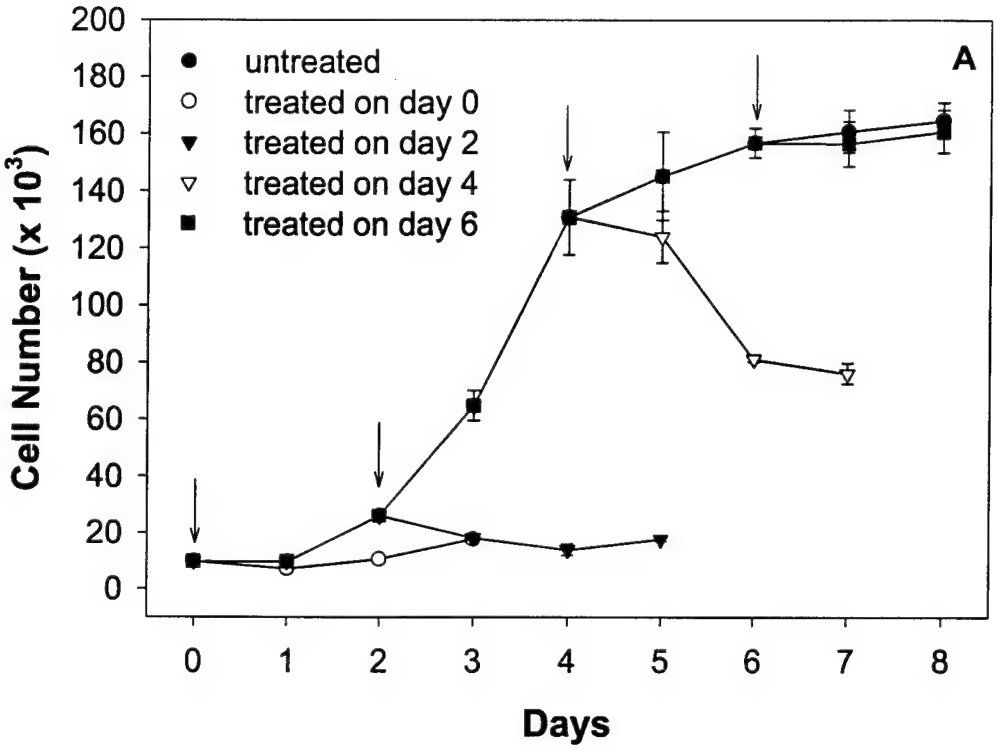
150
120
90
60
30
0
Day 0
Day 3
Day 6


A
Cell No.
CPM
Cell Number (x10^3)
^{3}H-Thymidine CPM (x10^3)
140
120
100
80
60
40
20
0
40
35
30
25
20
15
10
5
0
0
25
50
100
200
VEGI (ng/ml)


B
Hours
24
48
72
VEGI
-
+
-
+
-
+
ppRB
pRB
c-Myc
β-actin


A
untreated
treated on day 0
treated on day 2
treated on day 4
treated on day 6
Cell Number (x 10³)
Days
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20
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60
80
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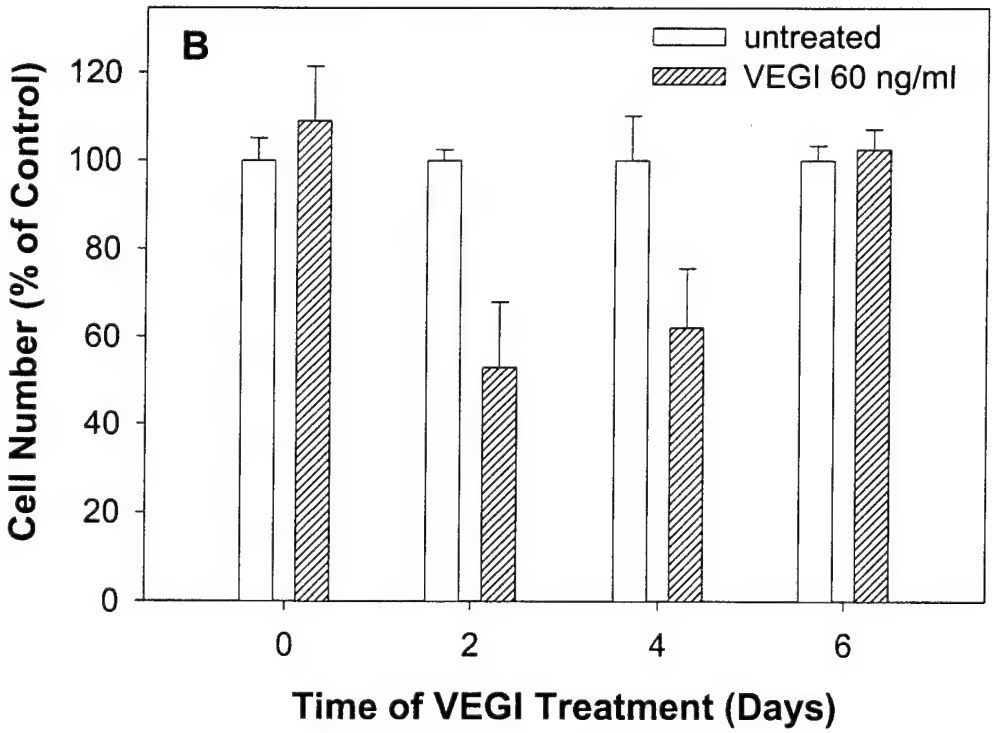
200
0
1
2
3
4
5
6
7
8


B
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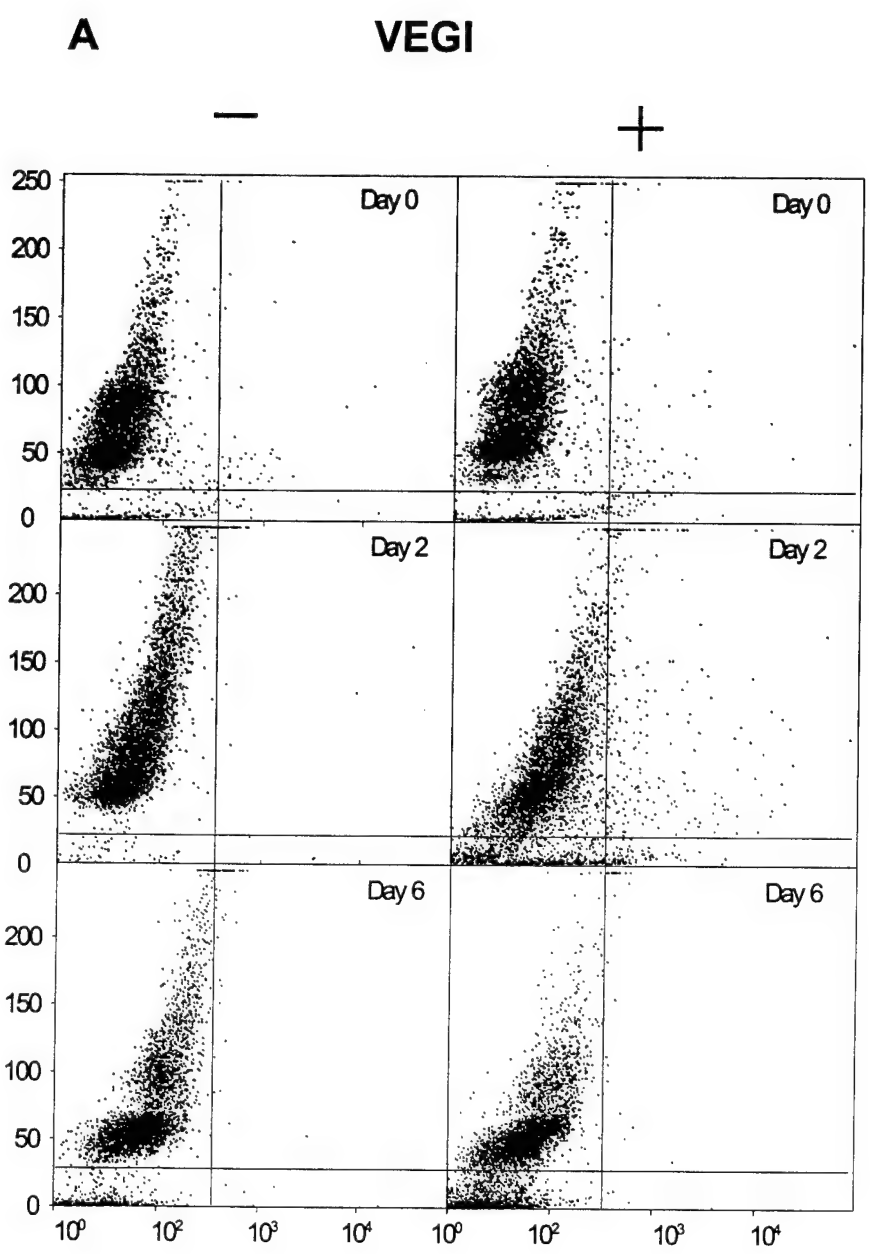
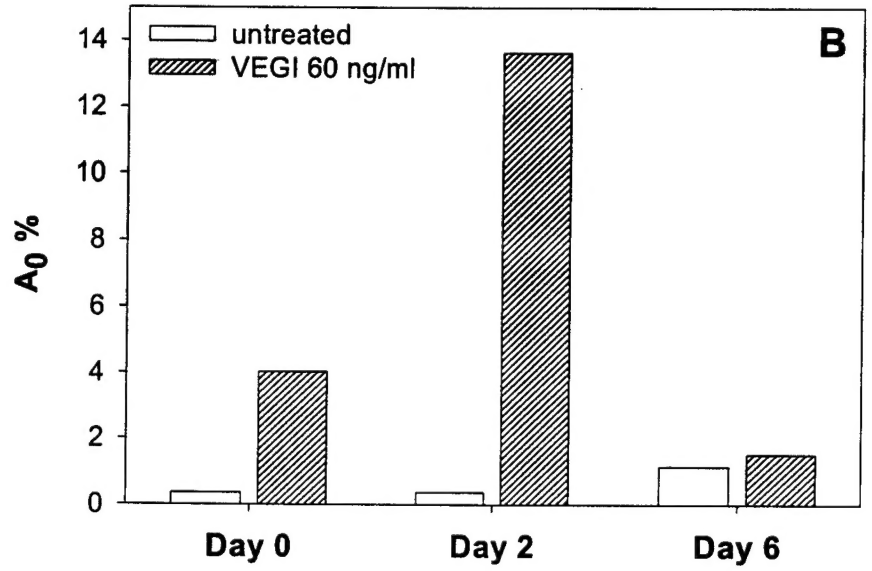
VEGI 60 ng/ml
Cell Number (% of Control)
Time of VEGI Treatment (Days)
0
20
40
60
80
100
120
0
2
4
6


A
VEGI
—
+
Day 0
Day 0
Day 2
Day 2
Day 6
Day 6
250
200
150
100
50
0
10^0
10^2
10^3
10^4


B
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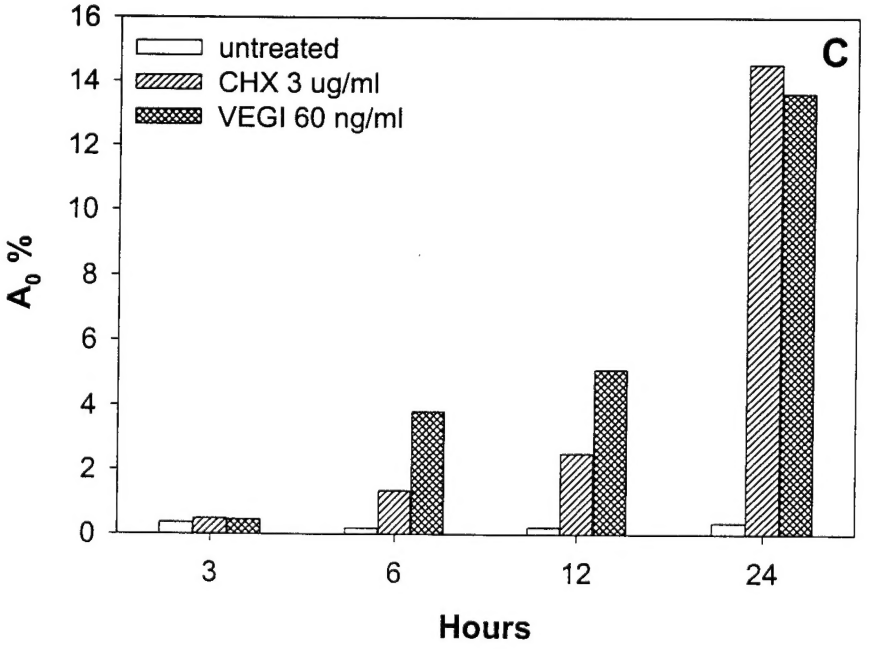
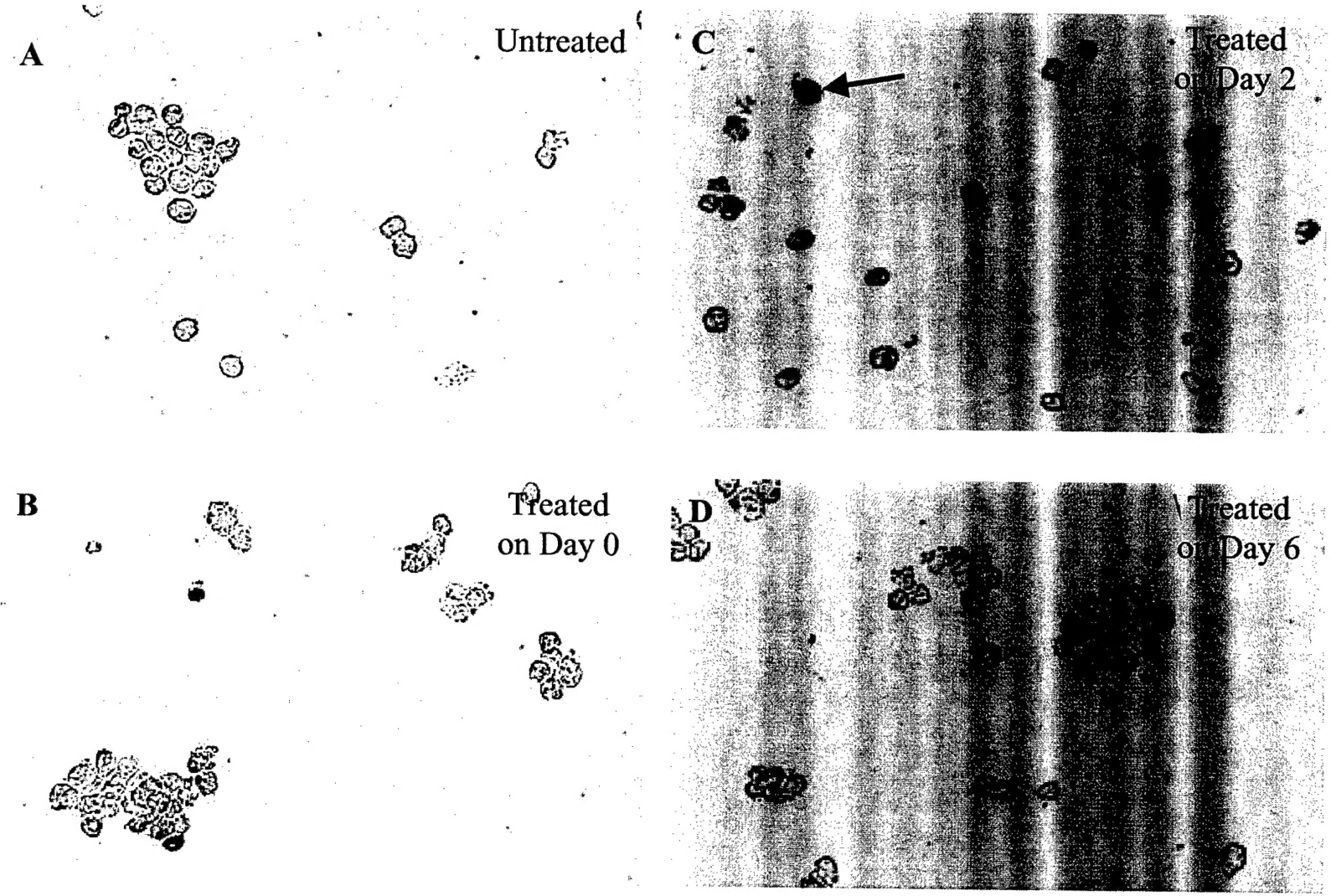
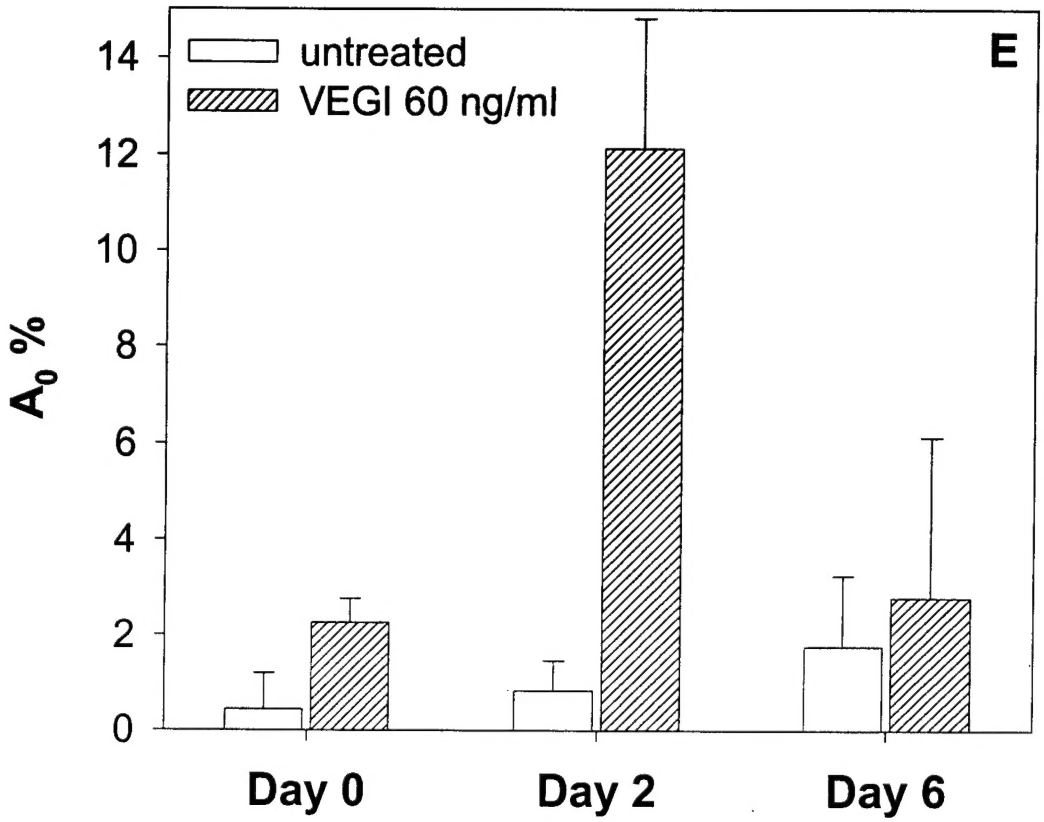
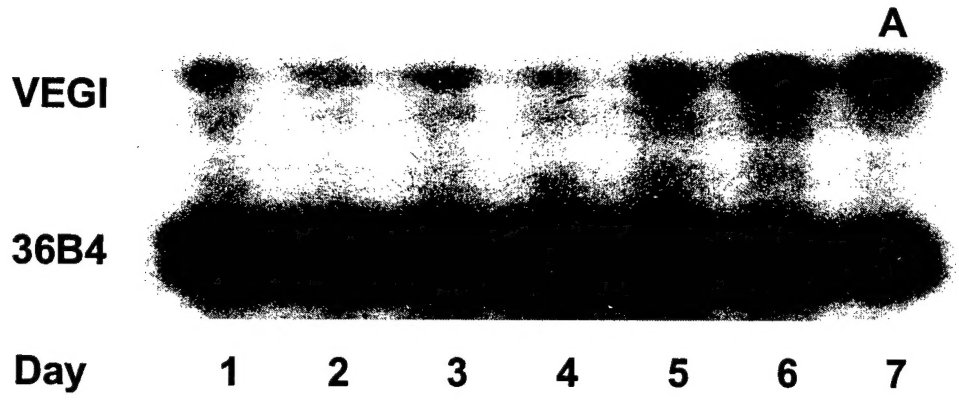
VEGI 60 ng/ml
A_0 %
0
2
4
6
8
10
12
14
Day 0
Day 2
Day 6

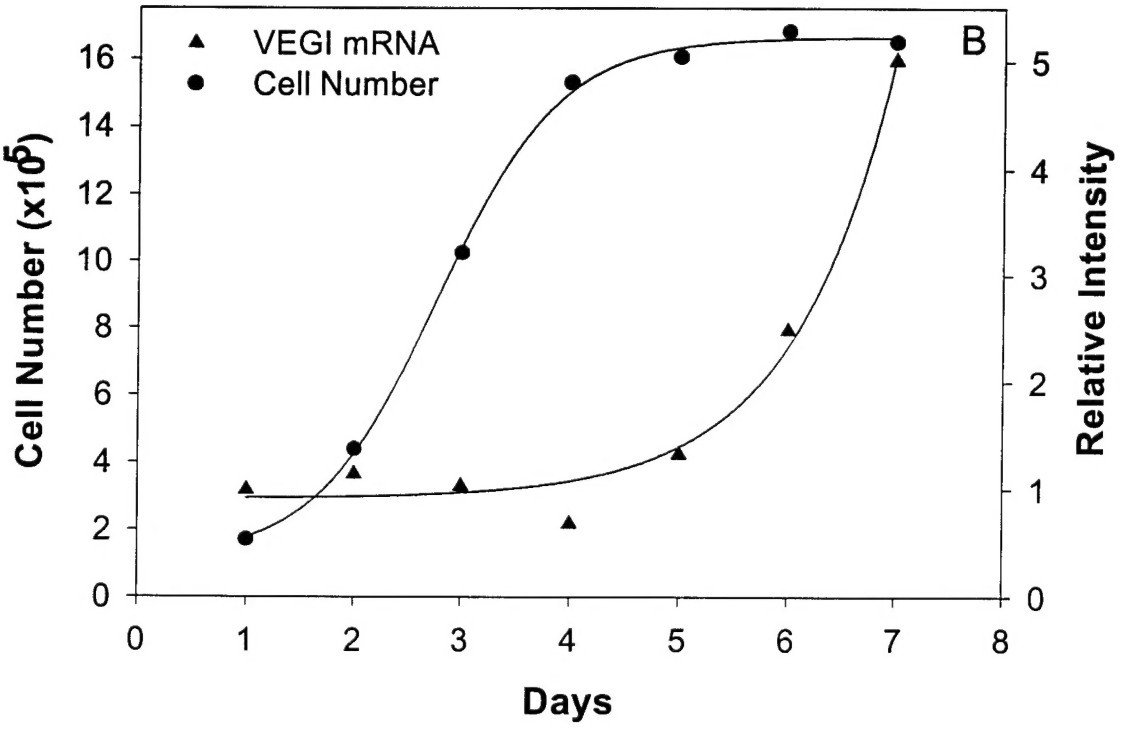

C
untreated
CHX 3 ug/ml
VEGI 60 ng/ml
A_0 %
0
2
4
6
8
10
12
14
16
3
6
12
24
Hours

Yu etal Fig 5ABCD




untreated
VEGI 60 ng/ml
E
14
12
10
8
6
4
2
0
A_0 %
Day 0
Day 2
Day 6


A
VEGI
36B4
Day
1
2
3
4
5
6
7


VEGI mRNA
Cell Number
B
Cell Number (x10⁵)
Relative Intensity
Days
0
2
4
6
8
10
12
14
16
1
3
5
7